# PCT

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification [5] : | | (11) International Publication Number: | WO 92/22641 |
|---|---|---|---|
| C12N 7/00, A61K 39/12 | A1 | (43) International Publication Date: | 23 December 1992 (23.12.92) |

(21) International Application Number: PCT/US92/05107

(22) International Filing Date: 12 June 1992 (12.06.92)

(30) Priority data:
715,921      14 June 1991 (14.06.91)    US
897,382      11 June 1992 (11.06.92)    US

(71) Applicant: VIROGENETICS CORPORATION [US/US]; 465 Jordan Road, Rensselaer Technology Park, Troy, NY 12180 (US).

(72) Inventors: PAOLETTI, Enzo ; 297 Murray Avenue, Delmar, NY 12054 (US). TARTAGLIA, James ; 7 Christina Drive, Schenectady, NY 12303 (US). COX, William, I. ; 1 Washington Place, Troy, NY 12180 (US).

(74) Agents: FROMMER, William, S. et al.; Curtis, Morris & Safford, 530 Fifth Avenue, New York, NY 10036 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
*With international search report.*

(54) Title: IMMUNODEFICIENCY VIRUS RECOMBINANT POXVIRUS VACCINE

(57) Abstract

What is described is a recombinant poxvirus, such as vaccinia virus, canarypox virus and fowlpox virus, having enhanced safety and containing foreign DNA from an immunodeficiency virus, such as human immunodeficiency virus and simian immunodeficiency virus. What is also described is an immunogenic composition which induces an immunological response against immunodeficiency virus infections when administered to a host.

B12

# IMMUNODEFICIENCY VIRUS RECOMBINANT POXVIRUS VACCINE

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of
copending application Serial No. 07/715,921, filed June
5   14, 1991, incorporated herein by reference.  Reference is
also made to copending application Serial No. 07/847,951,
filed March 6, 1992, also incorporated herein by
reference.

## FIELD OF THE INVENTION

10          The present invention relates to a modified
poxvirus and to methods of making and using the same.
More in particular, the invention relates to recombinant
poxvirus, which virus expresses gene products of an
immunodeficiency virus gene, and to immunogenic
15  compositions which induce an immunological response
against immunodeficiency virus infections when
administered to a host.

Several publications are referenced in this
application.  Full citation to these references is found
20  at the end of the specification preceding the claims.
These references describe the state-of-the-art to which
this invention pertains.

## BACKGROUND OF THE INVENTION

Vaccinia virus and more recently other
25  poxviruses have been used for the insertion and
expression of foreign genes.  The basic technique of
inserting foreign genes into live infectious poxvirus
involves recombination between pox DNA sequences flanking
a foreign genetic element in a donor plasmid and
30  homologous sequences present in the rescuing poxvirus
(Piccini et al., 1987).

Specifically, the recombinant poxviruses are
constructed in two steps known in the art and analogous
to the methods for creating synthetic recombinants of the
35  vaccinia virus described in U.S. Patent Nos. 5,110,587,
4,769,330, 4,772,848 and 4,603,112, the disclosures of
which are incorporated herein by reference.  In this

regard, reference is also made to copending U.S. applications Serial Nos. _____, filed May 4, 1992 and 537,890, filed June 14, 1990, also incorporated herein by reference.

5      First, the DNA gene sequence to be inserted into the virus, particularly an open reading frame from a non-pox source, is placed into an *E. coli* plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the DNA

10  gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA containing a nonessential locus. The

15  resulting plasmid construct is then amplified by growth within *E. coli* bacteria (Clewell, 1972) and isolated (Clewell et al., 1969; Maniatis et al., 1986).

      Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell

20  culture, e.g. chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively gives a poxvirus modified by the presence, in a nonessential region of its genome, of foreign DNA sequences. The term

25  "foreign" DNA designates exogenous DNA, particularly DNA from a non-pox source, that codes for gene products not ordinarily produced by the genome into which the exogenous DNA is placed.

      Genetic recombination is in general the

30  exchange of homologous sections of DNA between two strands of DNA. In certain viruses RNA may replace DNA. Homologous sections of nucleic acid are sections of nucleic acid (DNA or RNA) which have the same sequence of nucleotide bases.

35      Genetic recombination may take place naturally during the replication or manufacture of new viral genomes within the infected host cell. Thus, genetic

recombination between viral genes may occur during the
viral replication cycle that takes place in a host cell
which is co-infected with two or more different viruses
or other genetic constructs. A section of DNA from a
5    first genome is used interchangeably in constructing the
section of the genome of a second co-infecting virus in
which the DNA is homologous with that of the first viral
genome.

         However, recombination can also take place
10   between sections of DNA in different genomes that are not
perfectly homologous. If one such section is from a
first genome homologous with a section of another genome
except for the presence within the first section of, for
example, a genetic marker or a gene coding for an
15   antigenic determinant inserted into a portion of the
homologous DNA, recombination can still take place and
the products of that recombination are then detectable by
the presence of that genetic marker or gene in the
recombinant viral genome.

20         Successful expression of the inserted DNA
genetic sequence by the modified infectious virus
requires two conditions. First, the insertion must be
into a nonessential region of the virus in order that the
modified virus remain viable. The second condition for
25   expression of inserted DNA is the presence of a promoter
in the proper relationship to the inserted DNA. The
promoter must be placed so that it is located upstream
from the DNA sequence to be expressed.

         In recent years much attention within the field
30   of medical virology has been focused on the escalating
incidence of acquired immunodeficiency syndrome (AIDS)
caused by human immunodeficiency virus (HIV). HIV-1 is a
member of the virus family, Retroviridae, and more
specifically of the Lentivirus subfamily. This viral
35   system along with other related viruses such as HIV-2 and
simian immunodeficiency virus (SIV) have been scrutinized
with respect to their molecular biology, immunology, and

4

pathogenesis in an effort to develop safe and efficacious
vaccines and antiviral therapies. Two major obstacles,
however, have haunted the design of HIV vaccine
candidates. For one, there exists considerable sequence
5    heterogeneity among HIV isolates, and secondly, there
exists a lack of information pertaining to protective
immunity. Infected individuals develop antibody
(Starcich et al., 1986; Weiss et al., 1985) and $CD8^+$-T
cell (Walker et al., 1987; Plata et al., 1987) responses
10   to HIV but are not protected since they eventually
develop AIDS.

Vaccinia virus has been used extensively to
express a number of HIV gene products to investigate
their biochemical, functional and immunological
15   properties, particularly cell-mediated responses. Using
these specific reagents, cell-mediated cytotoxic
activities have been demonstrated in seropositive
individuals towards the envelope (Riviere et al., 1989;
Walker et al., 1987; Koup et al., 1989), core proteins
20   (gag) (Riviere et al., 1989; Koup et al., 1989, Nixon et
al., 1988) pol (Walker et al., 1989; Walker et al.,
1988), and the nef (Riviere et al., 1989) gene products.
Additionally, vaccinia virus recombinants containing HIV
genes have been shown to elicit both cell-mediated and
25   humoral immune responses in small laboratory animals
(Chakrabarti et al., 1986; Hu et al., 1986; Rautman et
al., 1989; Michel et al., 1988), macaques (Zarling et
al., 1986), chimpanzees (Hu et al., 1987) and,
significantly, humans (Zagury et al., 1988; Koff et al.,
30   1989). To date, limited vaccination/protection studies
in primates have been reported with vaccinia virus
recombinants expressing gene products from HIV and
related viruses.

Vaccination of primates with a recombinant
35   vaccinia virus expressing the envelope glycoprotein from
AIDS-causing retroviruses have elicited humoral and cell-
mediated immune responses and, more significantly, have

5

protected against HIV infection (Zagury et al., 1988; Hu
et al., 1989). In the results described by Hu et al., a
vaccinia virus recombinant expressing the envelope
glycoprotein from simian AIDS retrovirus (SRV-2) was used
5    to vaccinate pig-tailed macaques (*Macaca nemestrina*).
All immunized animals developed both SRV-specific cell-
mediated and humoral immune responses, including
neutralizing antibodies and antibodies which mediate ADCC
towards SRV-2 infected cells. Animals were challenged
10   intravenously with $5 \times 10^3$ TCID$_{50}$ of SRV-2/W. The
challenged control animals (non-vaccinated) became
viremic by two weeks post-challenge and those that did
not seroconvert died by seven weeks post-challenge.
Significantly, all of the challenged animals which were
15   previously inoculated with the vaccinia virus-env
recombinant remained healthy, virus-free and seropositive
exclusively against the envelope antigen.

In a pilot experiment in humans, HIV
seronegative individuals were vaccinated with a vaccinia
20   virus/HIV-1 envelope recombinant. This primary
inoculation resulted in weak immunological responses
(Zagury et al., 1988). These primary responses were
subsequently boosted with various protocols. The use of
an intravenous injection of paraformaldehyde-fixed
25   autologous cells infected *in vitro* with the vaccinia
virus-HIV recombinant, however, provided the most
significant booster effect. With this immunization
protocol, anamnestic immune responses were achieved
against the envelope antigen consisting of group-specific
30   neutralizing antibodies, cytotoxic T-lymphocytes and
delayed-type hypersensitivity (Zagury et al., 1988). In
clinical trials performed in the United States using a
recombinant vaccinia virus expressing the HIV env gene
(HIVAV-1e; Bristol-Meyers), individuals receiving the
35   recombinant mounted T-cell proliferative responses to HIV
(Koff et al., 1989). The intensity of these responses,
however, was affected by prior exposure to vaccinia

6

virus. Consequently, individuals immune to vaccinia
virus mounted a weak and transient T-cell response
whereas in individuals not immune to vaccinia virus, a
strong response was observed towards the HIV envelope
5   antigen (reviewed by Koff et al., 1989). These results
are encouraging and have provided evidence that an immune
state can be obtained in man prior to HIV exposure using
a poxvirus-based immunization vehicle.
        An intriguing potential in terms of HIV-1
10  vaccinology is provided by expression of the HIV-1 gag
products by vaccinia virus either alone or in combination
with the envelope glycoprotein. Several laboratories
have demonstrated that expression of gag or gag/pol
sequences by vaccinia virus results in the production of
15  non-infectious particles (Hu et al., 1990; Shioda et al.,
1990; Karacostas et al., 1989). Analysis of these
vaccinia virus recombinant infected cells by electron
microscopy revealed retrovirus-like particles budding
from the plasma membrane and extracellular forms which
20  were indistinguishable from particles observed in HIV-1
infected cells. Biochemically, these particles are also
similar to native HIV-1 particles. Moreover, rodents and
chimpanzees inoculated with a vaccinia virus-HIV gag
recombinant generated both humoral and cell-mediated
25  immune responses to the HIV-1 core antigens (Hu et al.,
1990).
        The co-expression of the HIV-1 envelope
glycoprotein with the core proteins in mammalian cells by
vaccinia virus also resulted in the assembly and release
30  of HIV-1 particles (Haffar et al., 1990). These typical
type C-retrovirus particles contained both the envelope
glycoproteins (gp120 and gp41) and the gag proteins (p24,
p17, p55, and p39). These proteins were also described
as being present in these recombinant-made particles in
35  the same ratio as observed in HIV-1 virions. The
production of these non-infectious HIV particles either
in purified form or synthesized in a vaccinee upon

7

inoculation with a multivalent vaccinia virus recombinant
may provide valuable "whole-virus" vaccinating agents
against HIV.  This is especially appealing since
protection against the lentivirus, SIV, has been
5    demonstrated using whole inactivated virus preparations
(Murphy-Corb et al., 1989; Derosiers et al., 1989).

          The above reviewed examples demonstrate the
potential use of vaccinia virus recombinants expressing
HIV antigens to induce pertinent immunological responses
10   necessary for protection.  A major concern, however,
about the use of live viral vaccines is the issue of
safety.  Rare complications from immunization with
vaccinia virus have been documented, particularly in
immunocompromised individuals, and have raised some
15   objections for their acceptance as vaccine candidates
(Behbehani, 1983; La~e et al., 1969).  Recently, however,
significant strides  ve been made in understanding viral
virulence factors with the hope of modifying strains for
use as immunization vehicles (Tartaglia et al., 1990).
20   With more relevance to the development of vaccinia virus-
based HIV vaccine candidates for the immunoprophylaxis
and immunotherapy of AIDS, the genes responsible for the
productive replication of vaccinia virus in human cell
systems have been identified (Gillard et al., 1986;
25   Perkus et al., 1990).  Potential use of vaccinia virus
vectors devoid of these genes provide a non-replicating
vector vaccine candidate that may present appropriate HIV
antigens in a fashion that elicits a protective immune
response.

30          Another approach towards the generation of ~afe
and effective poxvirus based HIV vaccine candidates
utilize avipoxvirus vectors (i.e. canarypoxvirus and
fowlpoxvirus) to express pertinent HIV antigens.  These
viruses are naturally host-restricted and only
35   productively replicate in avian species.  Therefore,
there exists a built-in safety factor for their use in
humans, particularly immunocompromised individuals.  In

8

this regard, these viruses have been engineered to
express the rabies G glycoprotein and have been
demonstrated to protect various nonavian species from
live rabies challenge (Taylor et al., 1988a; 1991).

5          It can thus be appreciated that provision of an
immunodeficiency virus recombinant poxvirus, and of an
immunogenic composition which induces an immunological
response against immunodeficiency virus infections when
administered to a host, particularly a composition having
10   enhanced safety, would be a highly desirable advance over
the current state of technology.

## OBJECTS OF THE INVENTION

           It is therefore an object of this invention to
provide recombinant poxviruses, which viruses express
15   gene products of an immunodeficiency virus, and to
provide a method of making such recombinant poxviruses.

           It is an additional object of this invention to
provide for the cloning and expression of
immunodeficiency virus coding sequences, particularly
20   human immunodeficiency virus (HIV) and simian
immunodeficiency virus (SIV) coding sequences in a
poxvirus vector.

           It is another object of this invention to
provide an immunological composition having enhanced
25   safety and which is capable of inducing an immunological
response against immunodeficiency virus infections when
administered to a host.

           These and other objects and advantages of the
present invention will become more readily apparent after
30   consideration of the following.

## STATEMENT OF THE INVENTION

           In one aspect, the present invention relates to
a recombinant poxvirus containing therein a DNA sequence
from an immunodeficiency virus, particularly HIV or SIV,
35   in a nonessential region of the poxvirus genome.  The
poxvirus is advantageously a vaccinia virus or an avipox
virus, such as fowlpox virus or canarypox virus.

According to the present invention, the
recombinant poxvirus expresses gene products of the
foreign immunodeficiency virus gene, particularly an HIV
or SIV gene.

5           In another aspect, the present invention
relates to a vaccine for inducing an immunological
response in a host animal inoculated with the vaccine,
said vaccine including a carrier and a recombinant
poxvirus containing, in a nonessential region thereof,
10  DNA from an immunodeficiency virus, particularly HIV or
SIV.  The poxvirus used in the vaccine according to the
present invention is advantageously a vaccinia virus or
an avipox virus, such as fowlpox virus or canarypox
virus.

15          **BRIEF DESCRIPTION OF THE DRAWINGS**
        A better understanding of the present invention
will be had by referring to the accompanying drawings, in
which:
        FIG. 1 schematically shows a method for the
20  construction of plasmid pSD460 for deletion of thymidine
kinase gene and generation of recombinant vaccinia virus
vP410;
        FIG. 2 schematically shows a method for the
construction of plasmid pSD486 for deletion of
25  hemorrhagic region and generation of recombinant vaccinia
virus vP553;
        FIG. 3 schematically shows a method for the
construction of plasmid pMP494Δ for deletion of ATI
region and generation of recombinant vaccinia virus
30  vP618;
        FIG. 4 schematically shows a method for the
construction of plasmid pSD467 for deletion of
hemagglutinin gene and generation of recombinant vaccinia
virus vP723;
35          FIG. 5 schematically shows a method for the
construction of plasmid pMPCSK1Δ for deletion of gene

cluster [C7L - K1L] and generation of recombinant
vaccinia virus vP804;

      FIG. 6 schematically shows a method for the
construction of plasmid pSD548 for deletion of large

5   subunit, ribonucleotide reductase and generation of
recombinant vaccinia virus vP866 (NYVAC);

      FIG. 7 shows the cytotoxic response of spleen
cells of mice and immunized with vaccinia virus or
canarypox virus vectors (NYVAC, ALVAC) or with vaccinia

10  virus or canarypox virus recombinants expressing HIV III
B env (vP911, vCP112);

      FIG. 8 shows the specificity of cytotoxic T
lymphocyte antigen receptor for the HIV II B
hypervariable V3 loop of gp120, but not for the V3 loop

15  of HIV MN or SF2;

      FIG. 9 shows the antibody responses to HIV III
B gp120 of mice immunized with vectors (NYVAC, ALVAC) or
with vaccinia virus recombinant vP911 or canarypox
recombinant vCP112 expressing HIV-1 env (inverted

20  triangle indicates time of administration of second
inoculation);

      FIG. 10 shows the sensitivity of the cytotoxic
effector cells from the spleens of mice immunized with
vCP112 to antibodies against cytotoxic T lymphocyte cell

25  surface antigens Thy 1.2 and Lyt 2.2; and

      FIG. 11 shows percent cytotoxicity versus *in
vitro* stimulation of PBMC.

### DETAILED DESCRIPTION OF THE INVENTION

      A better understanding of the present invention

30  and of its many advantages will be had from the following
examples, given by way of illustration.

      In the Examples, the following methods and
materials are employed.

### DNA Cloning and Synthesis

35       Plasmids were constructed, screened and grown
by standard procedures (Maniatis et al., 1982; Perkus et
al., 1985; Piccini et al., 1987). Restriction

11

endonucleases were obtained from GIBCO/BRL, Gaithersburg,
MD; New England Biolabs, Beverly, MA; and Boehringer
Mannheim Biochemicals, Indianapolis, IN. Klenow fragment
of *E. coli* polymerase was obtained from Boehringer

5  Mannheim Biochemicals. BAL-31 exonuclease and phage T4
DNA ligase were obtained from New England Biolabs. The
reagents were used as specified by the various suppliers.
        Synthetic oligodeoxyribonucleotides were
prepared on a Biosearch 8750 or Applied Biosystems 380B

10  DNA synthesizer as previously described (Perkus et al.,
1989). DNA sequencing was performed by the dideoxy-chain
termination method (Sanger et al., 1977) using Sequenase
(Tabor et al., 1987) as previously described (Guo et al.,
1989). DNA amplification by polymerase chain reaction

15  (PCR) for sequence verification (Engelke et al., 1988)
was performed using custom synthesized oligonucleotide
primers and GeneAmp DNA amplification Reagent Kit (Perkin
Elmer Cetus, Norwalk, CT) in an automated Perkin Elmer
Cetus DNA Thermal Cycler. Excess DNA sequences were

20  deleted from plasmids by restriction endonuclease
digestion followed by limited digestion by BAL-31
exonuclease and mutagenesis (Mandecki, 1986) using
synthetic oligonucleotides.

**Cells, Virus, and Transfection**

25              The origins and conditions of cultivation of
the Copenhagen strain of vaccinia virus has been
previously described (Guo et al., 1989). Generation of
recombinant virus by recombination, *in situ* hybridization
of nitrocellulose filters and screening for B-

30  galactosidase activity are as previously described
(Panicali et al., 1982; Perkus et al., 1989).
        The parental canarypox virus (Rentschler
strain) is a vaccinal strain for canaries. The vaccine
strain was obtained from a wild type isolate and

35  attenuated through more than 200 serial passages on chick
embryo fibroblasts. A master viral seed was subjected to
four successive plaque purifications under agar and one

12

plaque clone was amplified through five additional
passages after which the stock virus was used as the
parental virus in *in vitro* recombination tests.   The
plaque purified canarypox isolate is designated ALVAC.

5          The strain of fowlpox virus (FPV) designated
FP-1 has been described previously (Taylor et al.,
1988a).   It is an attenuated vaccine strain useful in
vaccination of day old chickens.   The parental virus
strain Duvette was obtained in France as a fowlpox scale
10   from a chicken.   The virus was attenuated by
approximately 50 serial passages in chicken embryonated
eggs followed by 25 passages on chicken embryo fibroblast
cells.   The virus was subjected to four successive plaque
purifications.   One plaque isolate was further amplified
15   in primary CEF cells and a stock virus, designated as
TROVAC, established.

          NYVAC, ALVAC and TROVAC viral vectors and their
derivatives were propagated as described previously
(Piccini et al., 1987; Taylor et al., 1988a,b).   Vero
20   cells and chick embryo fibroblasts (CEF) were propagated
as described previously (Taylor et al., 1988a,b).   P815
murine mastocytoma cells (H-2$^d$) were obtained from ATCC
(#TIB64) and maintained in Eagles MEM supplemented with
10% fetal bovine serum CFBS and 100 IU/ml penicillin and
25   100 $\mu$g streptomycin per ml.

**Mice**

          Female BALB/cJ (H-2$^d$) mice were purchased from
The Jackson Laboratories (Bar Harbor, ME) and maintained
on mouse chow and water ad libitum.   All mice were used
30   between the ages of 6 and 15 weeks of age.

**Media**

          Assay Medium for immunological assays was
comprised of RPMI 1640 medium supplemented with 10% FBS,
4 mM L-glutamine, 20 mM HEPES
35   (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate), 5x10$^{-5}$
M 2-mercaptoethanol, 100 IU penicillin per ml, and 100
$\mu$g/ml streptomycin.   Stim Medium was comprised of Eagle's

13

Minimum Essential Medium supplemented with 10% FBS, 4 mM
L-glutamine, $10^{-4}$ M 2-mercaptoethanol, 100 IU penicillin
per ml, and 100 μg streptomycin per ml.

**Radioimmunoprecipitation Analysis**

5      Cell monolayers were infected at 10 PFU/cell in
modified Eagle's methionine-free medium (MEM met-).  At 2
hours post-infection, 20 uCi/ml of [$^{35}$S]-methionine were
added in MEM (-met) containing 2% dialysed fetal bovine
serum (Flow).  Cells were harvested at 15 hrs post-

10   infection by resuspending them in lysis buffer (150 mM
NaCl, 1mM EDTA pH 8, 10 mM Tris-HCl (pH 7.4), 0.2 mg/ml
PMSF, 1% NP40, 0.01% Na Azide) and 50 μl aprotinin,
scraped into eppendorf tubes and the lysate was clarified
by spinning 20 minutes at 4°C.  One third of the

15   supernatant of a 60 mm diameter Petri dish was incubated
with 1 μl normal human serum and 100 μl of protein A-
Seraphose CL-4B (SPA) (Pharmacia) for 2 hours at room
temperature.  After spinning for 1 minute, the
supernatant was incubated for 90 min at 4°C with immune

20   sera specifically recognizing HIV-1, HIV-2, or SIV
proteins and 100 μl SPA.

The pellet was washed four times with lysis
buffer and two times with lithium chloride/urea buffer
(0.2 M LiCl, 2 M urea, 10 mM Tris-HCl pH 8) and the

25   precipitated proteins were dissolved in 60 μl Laemmli
buffer (Laemmli, 1970).  After heating for 5 minutes at
100°C and spinning for 1 minute, proteins were resolved
on a SDS 10% polyacrylamide gel and fluorographed.

**Inoculations**

30      Mice were intravenously inoculated with 5x10$^7$
plaque forming units (PFU) in 0.1 ml of phosphate-
buffered saline via the lateral tail vein.

**Spleen Cell Preparations**

Following euthanasia by cervical dislocation,

35   the spleens of mice were aseptically transferred to a
sterile plastic bag containing Hank's Balanced Salt
Solution.  Individual spleens or pooled spleens from a

14

single experimental group were processed to single cell
suspensions by a 1 minute cycle in a Stomacher blender.
The spleen cell suspensions were washed several times in
either Assay Medium or Stim Medium, as appropriate.  The

5   spleen cells were enumerated by the use of a Coulter
Counter or by trypan blue dye exclusion using a
hemacytometer and microscope.

Sera

Mice were lightly anesthetized with ether and

10  blood was collected from the retroorbital plexus.  Blood
from mice comprising an experimental group was pooled and
allowed to clot.  The serum was collected and stored at -
70°C until use.

*In Vitro* Stimulation for the Generation of Secondary

15  Cytotoxic T Lymphocytes (CTL)

The pooled spleen cells from the various
experimental groups (responders) were diluted to $5 \times 10^6$/ml
in Stim Medium.  The spleen cells from syngeneic, naive
mice (stimulators) were diluted to $1 \times 10^7$ cells per ml and

20  infected for 1 hour in tissue culture medium containing
2% FBS at 37°C with the appropriate poxvirus at an m.o.i.
of 25 PFU/cell.  Following infection, the stimulator
cells were washed several times in Stim Medium and
diluted to $1 \times 10^6$ cells per ml with Stim Medium.  Five mls

25  of stimulator cells and 5 mls of responder cells were
added to a 25 cm$^3$ tissue culture flask and incubated
upright at 37°C, in 5% $CO_2$ for 5 days.  On the day of the
assay, the spleen cells were washed several times in
Assay Medium and counted on a hemacytometer in trypan

30  blue with the use of a microscope.

Target Cell Preparation

For poxvirus specific CTL activity, tissue
culture cells were infected overnight by incubation at
$1 \times 10^7$ cells per ml in tissue culture medium containing 2%

35  FBS at an m.o.i. of 25 PFU/cell for 1 hour at 37°C.
Following incubation, the cells were diluted to between 1
- $2 \times 10^6$ cells per ml with tissue culture medium

15

containing 10% FBS and further incubated at 37°C, in 5% $CO_2$ until use.  For HIV specific CTL activity, tissue culture cells were incubated overnight with 20 $\mu$g/ml of peptide HBX2 (American Biotechnologies, Cambridge, MA),

5  SF2 (American Biotechnologies, Cambridge, MA) or MN (American Biotechnologies, Cambridge, MA) corresponding to the V3 loop region of gp120 of HIV-1 isolates $III_B$, SF2, and MN, respectively.  On the day of the assay, the targets were washed several times by centrifugation in

10  Assay Medium.  After the final wash, the cells were resuspended in approximately 100 $\mu$Ci of $Na_2{}^{51}CrO_4$ ($^{51}$Cr). Following incubation at 37°C for 1 hr, the cells were washed at least 3 times in Assay Medium by centrifugation, counted on a hemacytometer, and diluted

15  to $1x10^5$/ml in Assay Medium.

## Cytotoxicity Assays

For primary CTL assays, freshly prepared spleen cells were diluted with Assay Medium to $1x10^7$ cells per ml.  For secondary CTL assays (following either *in vivo*

20  inoculation or *in vitro* stimulation), the spleen cells were diluted to $2x10^6$/ml in Assay Medium.  One tenth ml of spleen cell suspension was added to $^{51}$Cr labelled target cells in the wells of a 96 well, round-bottom microtiter plate (EXP).  In most cases, the spleen cells

25  being assayed for primary CTL activity were further 2-fold diluted in the wells of the microtiter plate prior to the addition of the target cells.  As a measure of spontaneous release of $^{51}$Cr (SR), target cells were incubated in only Assay Medium.  To determine the maximum

30  release of $^{51}$Cr (MAX), target cells were deliberately lysed at the beginning of the assay by adding 0.1 ml of 10% sodium dodecyl sulfate to the appropriate wells.  To initiate the assay, the microtiter plates were centrifuged at 200 x g for 2 min and incubated for 4 or 5

35  hrs at 37°C, in 5% $CO_2$.  Following incubation, the culture supernatants of each well were collected using the Skatron Supernatant Collection System.  Released $^{51}$Cr

was determined by a Beckman 5500B gamma counter. The percent specific cytotoxicity was determined from the counts by the following formula:

$$\% \text{ CYTOTOXICITY} = (EXP-MAX)/(MAX-SR) \times 100$$

5 **Depletion of T Helper Cells and Cytotoxic T Lymphocytes Using Monoclonal Anti-CD4 and Monoclonal Anti-CD8**

Spleen cell suspensions were diluted to a density of $10^7/ml$ in cytotoxicity medium (RPMI 1640 containing 0.2% BSA and 5 mM HEPES) containing a 1:5

10 dilution of anti-CD4 (monoclonal antibody 172.4) or a 1:200 dilution of anti-CD8 (monoclonal antibody anti-Lyt 2.2) and a 1:16 dilution of Cedar Lane Low-Tox rabbit complement. Appropriate controls for the single components (complement, anti-CD4, anti-CD8) were

15 included.

**Anti-HIV-1 gp160 ELISA**

The wells of ELISA plates (Immulon II) were coated overnight at 4°C with 100 ng of purified HIV-1 gp160 (Immuno) in carbonate buffer, pH 9.6. The plates

20 were then washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST). The plates were then blocked for 2 hrs at 37°C with PBST containing 1% bovine serum albumin (BSA). After washing with PBST, sera were initially diluted 1:20 with PBST containing 0.1% BSA

25 (dilution buffer). The sera were further 2-fold serially diluted in the wells of the ELISA plate. The plates were incubated at 37°C for 2 hrs and washed with PBST. Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins (DAKO) was diluted 1:2000 in dilution

30 buffer and added to the wells of the ELISA plated and incubated at 37°C for 1 hour. After washing with PBST, OPD (o-phenylenediamine dihydrochloride) in substrate buffer was added and the color was allowed to develop at ambient temperature for about 20 minutes. The reaction

35 was extinguished by the addition of 2.5 M $H_2SO_4$. The absorbance at 490 nm was determined on a Bio-Tek EL-309 ELISA reader. The serum endpoint was defined as the

17

reciprocal of the dilution giving an absorbance value of 1.0.

**Lymphocyte Proliferation Assays**

Single cell suspensions of the spleen cells of
individual mice were diluted to $2x10^6$/ml in Assay Medium
and 0.1 ml were added to the wells of 96 well, flat-
bottom microtiter plates containing Assay Medium alone,
1, 5, or 10 μg of HIV-1 peptide T1, 1, 5, or 10 μg of
HIV-1 peptide T2, and 1 or 10 μg of purified HIV-1 gp160
(Immuno). The cells were incubated for 5 days at 37°C,
in 5% $CO_2$. To each well was added 1.0 μCi of
[$^3$H]-thymidine for the final 6 hrs of incubation and then
harvested onto Beckman Ready Filters using a Cambridge
PHD cell harvester. The filter disks were dry-counted in
a liquid scintillation counter.

STIMULATION INDEX = $CPMs_{EXP}/CPMs_{MEDIUM}$

**Example 1 – ATTENUATED VACCINIA VACCINE STRAIN NYVAC**

To develop a new vaccinia vaccine strain, NYVAC
(vP866), the Copenhagen vaccine strain of vaccinia virus
was modified by the deletion of six nonessential regions
of the genome encoding known or potential virulence
factors. The sequential deletions are detailed below.
All designations of vaccinia restriction fragments, open
reading frames and nucleotide positions are based on the
terminology reported in Goebel et al. (1990a,b).

The deletion loci were also engineered as
recipient loci for the insertion of foreign genes.

The regions sequentially deleted in NYVAC are
listed below. Also listed are the abbreviations and open
reading frame designations for the deleted regions
(Goebel et al., 1990a,b) and the designation of the
vaccinia recombinant (vP) containing all deletions
through the deletion specified:

(1) thymidine kinase gene (TK; J2R) vP410;

(2) hemorrhagic region (u; B13R + B14R) vP553;

(3) A type inclusion body region (ATI; A26L) vP618;

(4) hemagglutinin gene (HA; A56R) vP723;

18

     (5)  host range gene region (C7L - K1L) vP804; and

     (6)  large subunit, ribonucleotide reductase (I4L)

         vP866 (NYVAC).

     As described in following Examples, any or any

5 combination of these regions can be a site either alone

or in combination with other sites for inserting

exogenous DNA from an immunodeficiency virus,

immunodeficiency viruses or from such virus or viruses

and other exogenous DNA to obtain a useful recombinant.

10 **(1)**   **Construction of Plasmid pSD460 for Deletion of**

      **Thymidine Kinase Gene (J2R)**

     Referring now to FIG. 1, plasmid pSD406

contains vaccinia HindIII J (pos. 83359 - 88377) cloned

into pUC8.  pSD406 was cut with HindIII and PvuII, and

15 the 1.7 kb fragment from the left side of HindIII J

cloned into pUC8 cut with HindIII/SmaI, forming pSD447.

pSD447 contains the entire gene for J2R (pos. 83855 -

84385).  The initiation codon is contained within an

NlaIII site and the termination codon is contained within

20 an SspI site.  Direction of transcription is indicated by

an arrow in FIG. 1.

     To obtain a left flanking arm, a 0.8 kb

HindIII/EcoRI fragment was isolated from pSD447, then

digested with NlaIII and a 0.5 kb HindIII/NlaIII fragment

25 isolated.  Annealed synthetic oligonucleotides

MPSYN43/MPSYN44 (SEQ ID NO:1/SEQ ID NO:2)

```
                                  SmaI
MPSYN43  5'      TAATTAACTAGCTACCCGGG       3'
MPSYN44  3' GTACATTAATTGATCGATGGGCCCTTAA   5'
              NlaIII                   EcoRI
```

30

were ligated with the 0.5 kb HindIII/NlaIII fragment into

pUC18 vector plasmid cut with HindIII/EcoRI, generating

plasmid pSD449.

35      To obtain a restriction fragment containing a

vaccinia right flanking arm and pUC vector sequences,

pSD447 was cut with SspI (partial) within vaccinia

sequences and HindIII at the pUC/vaccinia junction, and a

2.9 kb vector fragment isolated.  This vector fragment

19

was ligated with annealed synthetic oligonucleotides
MPSYN45/MPSYN46 (SEQ ID NO:3/SEQ ID NO:4)

<u>HindIII</u> <u>SmaI</u>
MPSYN45    5'    AGCTTCCCGGGTAAGTAATACGTCAAGGAGAAAACGAA
5  MPSYN46    3'        AGGGCCCATTCATTATGCAGTTCCTCTTTTGCTT

                                    <u>NotI</u>               <u>SspI</u>
                       ACGATCTGTAGTTAGCGGCCGCCTAATTAACTAAT  3'
MPSYN45
10 MPSYN46                  TGCTAGACATCAATCGCCGGCGGATTAATTGATTA  5'

generating pSD459.

            To combine the left and right flanking arms
15  into one plasmid, a 0.5 kb <u>HindIII</u>/<u>SmaI</u> fragment was
    isolated from pSD449 and ligated with pSD459 vector
    plasmid cut with <u>HindIII</u>/<u>SmaI</u>, generating plasmid pSD460.
    pSD460 was used as donor plasmid for recombination with
    wild type parental vaccinia virus Copenhagen strain VC-2.
20  $^{32}$P labelled probe was synthesized by primer extension
    using MPSYN45 (SEQ ID NO:3) as template and the
    complementary 20mer oligonucleotide MPSYN47 (SEQ ID NO:5)
    (5' TTAGTTAATTAGGCGGCCGC 3') as primer.  Recombinant
    virus vP410 was identified by plaque hybridization.
25  (2)  <u>Construction of Plasmid pSD486 for Deletion of</u>
         <u>Hemorrhagic Region (B13R + B14R)</u>
            Referring now to FIG. 2, plasmid pSD419
    contains vaccinia <u>SalI</u> G (pos. 160,744-173,351) cloned
    into pUC8.  pSD422 contains the contiguous vaccinia <u>SalI</u>
30  fragment to the right, <u>SalI</u> J (pos. 173,351-182,746)
    cloned into pUC8.  To construct a plasmid deleted for the
    hemorrhagic region, <u>u</u>, B13R - B14R (pos. 172,549 -
    173,552), pSD419 was used as the source for the left
    flanking arm and pSD422 was used as the source of the
35  right flanking arm.  The direction of transcription for
    the <u>u</u> region is indicated by an arrow in FIG. 2.
            To remove unwanted sequences from pSD419,
    sequences to the left of the <u>NcoI</u> site (pos. 172,253)
    were removed by digestion of pSD419 with <u>NcoI</u>/<u>SmaI</u>
40  followed by blunt ending with Klenow fragment of *E. coli*
    polymerase and ligation generating plasmid pSD476.  A

20

vaccinia right flanking arm was obtained by digestion of
pSD422 with HpaI at the termination codon of B14R and by
digestion with NruI 0.3 kb to the right. This 0.3 kb
fragment was isolated and ligated with a 3.4 kb HincII
5 vector fragment isolated from pSD476, generating plasmid
pSD477. The location of the partial deletion of the
vaccinia u region in pSD477 is indicated by a triangle.
The remaining B13R coding sequences in pSD477 were
removed by digestion with ClaI/HpaI, and the resulting
10 vector fragment was ligated with annealed synthetic
oligonucleotides SD22mer/SD20mer (SEQ ID NO:6/SEQ ID
NO:7)

```
            ClaI          BamHI HpaI
SD22mer  5'  CGATTACTATGAAGGATCCGTT    3'
15 SD20mer  3'   TAATGATACTTCCTAGGCAA    5'
```

generating pSD479. pSD479 contains an initiation codon
(underlined) followed by a BamHI site. To place *E. coli*
Beta-galactosidase in the B13-B14 (u) deletion locus
20 under the control of the u promoter, a 3.2 kb BamHI
fragment containing the Beta-galactosidase gene (Shapira
et al., 1983) was inserted into the BamHI site of pSD479,
generating pSD479BG. pSD479BG was used as donor plasmid
for recombination with vaccinia virus vP410. Recombinant
25 vaccinia virus vP533 was isolated as a blue plaque in the
presence of chromogenic substrate X-gal. In vP533 the
B13R-B14R region is deleted and is replaced by Beta-
galactosidase.

        To remove Beta-galactosidase sequences from
30 vP533, plasmid pSD486, a derivative of pSD477 containing
a polylinker region but no initiation codon at the u
deletion junction, was utilized. First the ClaI/HpaI
vector fragment from pSD477 referred to above was ligated
with annealed synthetic oligonucleotides SD42mer/SD40mer
35 (SEQ ID NO:8/SEQ ID NO:9)

```
            ClaI          SacI       XhoI        HpaI
SD42mer  5' CGATTACTAGATCTGAGCTCCCCGGGCTCGAGGGATCCGTT
3'
SD40mer  3'   TAATGATCTAGACTCGAGGGGCCCGAGCTCCCTAGGCAA
40  5'
```

```
        BglII          SmaI           BamHI
```

generating plasmid pSD478. Next the EcoRI site at the
pUC/vaccinia junction was destroyed by digestion of

5   pSD478 with EcoRI followed by blunt ending with Klenow
fragment of *E. coli* polymerase and ligation, generating
plasmid pSD478E⁻. pSD478E⁻ was digested with BamHI and
HpaI and ligated with annealed synthetic
oligonucleotides HEM5/HEM6 (SEQ ID NO:10/SEQ ID NO:11)

10          BamHI EcoRI    HpaI
HEM5   5'   GATCCGAATTCTAGCT   3'
HEM6   3'        GCTTAAGATCGA   5'

generating plasmid pSD486. pSD486 was used as donor

15  plasmid for recombination with recombinant vaccinia virus
vP533, generating vP553, which was isolated as a clear
plaque in the presence of X-gal.

    (3) **Construction of Plasmid pMP494Δ for Deletion of ATI**
      **Region (A26L)**

20          Referring now to FIG. 3, pSD414 contains SalI B
cloned into pUC8. To remove unwanted DNA sequences to
the left of the A26L region, pSD414 was cut with XbaI
within vaccinia sequences (pos. 137,079) and with HindIII
at the pUC/vaccinia junction, then blunt ended with

25  Klenow fragment of *E. coli* polymerase and ligated,
resulting in plasmid pSD483. To remove unwanted vaccinia
DNA sequences to the right of the A26L region, pSD483 was
cut with EcoRI (pos. 140,665 and at the pUC/vaccinia
junction) and ligated, forming plasmid pSD484. To remove

30  the A26L coding region, pSD484 was cut with NdeI
(partial) slightly upstream from the A26L ORF (pos.
139,004) and with HpaI (pos. 137,889) slightly downstream
from the A26L ORF. The 5.2 kb vector fragment was
isolated and ligated with annealed synthetic

35  oligonucleotides ATI3/ATI4 (SEQ ID NO:12/SEQ ID NO:13)

```
          NdeI
ATI3   5'
TATGAGTAACTTAACTCTTTTGTTAATTAAAAGTATATTCAAAAAATAAGT
ATI4   3'
```

40  ACTCATTGAATTGAGAAAACAATTAATTTTCATATAAGTTTTTTATTCA

22

<u>Bgl</u>II <u>Eco</u>RI <u>Hpa</u>I
TATATAAATAGATCTGAATTCGTT  3' ATI3
ATATATTTATCTAGACTTAAGCAA  5' ATI4

5    reconstructing the region upstream from A26L and
     replacing the A26L ORF with a short polylinker region
     containing the restriction sites <u>Bgl</u>II, <u>Eco</u>RI and <u>Hpa</u>I,
     as indicated above.  The resulting plasmid was designated
     pSD485.  Since the <u>Bgl</u>II and <u>Eco</u>RI sites in the
10   polylinker region of pSD485 are not unique, unwanted
     <u>Bgl</u>II and <u>Eco</u>RI sites were removed from plasmid pSD483
     (described above) by digestion with <u>Bgl</u>II (pos. 140,136)
     and with <u>Eco</u>RI at the pUC/vaccinia junction, followed by
     blunt ending with Klenow fragment of *E. coli* polymerase
15   and ligation.  The resulting plasmid was designated
     pSD489.  The 1.8 kb <u>Cla</u>I (pos. 137,198)/<u>Eco</u>RV (pos.
     139,048) fragment from pSD489 containing the A26L ORF was
     replaced with the corresponding 0.7 kb polylinker-
     containing <u>Cla</u>I/<u>Eco</u>RV fragment from pSD485, generating
20   pSD492.  The <u>Bgl</u>II and <u>Eco</u>RI sites in the polylinker
     region of pSD492 are unique.
             A 3.3 kb <u>Bgl</u>II cassette containing the *E. coli*
     Beta-galactosidase gene (Shapira et al., 1983) under the
     control of the vaccinia 11 kDa promoter (Bertholet et
25   al., 1985; Perkus et al., 1990) was inserted into the
     <u>Bgl</u>II site of pSD492, forming pSD493KBG.  Plasmid
     pSD493KBG was used in recombination with rescuing virus
     vP553.  Recombinant vaccinia virus, vP581, containing
     Beta-galactosidase in the A26L deletion region, was
30   isolated as a blue plaque in the presence of X-gal.
             To generate a plasmid for the removal of Beta-
     galactosidase sequences from vaccinia recombinant virus
     vP581, the polylinker region of plasmid pSD492 was
     deleted by mutagenesis (Mandecki, 1986) using synthetic
35   oligonucleotide MPSYN177 (SEQ ID NO:14)
     (5' AAAATGGGCGTGGATTGTTAACTTTATATAACTTATTTTTTGAATATAC
     3').  In the resulting plasmid, pMP494Δ, vaccinia DNA
     encompassing positions [137,889 - 138,937], including the
     entire A26L ORF is deleted.  Recombination between the

pMP494Δ and the Beta-galactosidase containing vaccinia
recombinant, vP581, resulted in vaccinia deletion mutant
vP618, which was isolated as a clear plaque in the
presence of X-gal.

5    (4)   **Construction of Plasmid pSD467 for Deletion of
          Hemagglutinin Gene (A56R)**

         Referring now to FIG. 4, vaccinia <u>Sal</u>I G
restriction fragment (pos. 160,744-173,351) crosses the
<u>Hin</u>dIII A/B junction (pos. 162,539). pSD419 contains
10   vaccinia <u>Sal</u>I G cloned into pUC8. The direction of
transcription for the hemagglutinin (HA) gene is
indicated by an arrow in FIG. 4. Vaccinia sequences
derived from <u>Hin</u>dIII B were removed by digestion of
pSD419 with <u>Hin</u>dIII within vaccinia sequences and at the
15   pUC/vaccinia junction followed by ligation. The
resulting plasmid, pSD456, contains the HA gene, A56R,
flanked by 0.4 kb of vaccinia sequences to the left and
0.4 kb of vaccinia sequences to the right. A56R coding
sequences were removed by cutting pSD456 with <u>Rsa</u>I
20   (partial; pos. 161,090) upstream from A56R coding
sequences, and with <u>Eag</u>I (pos. 162,054) near the end of
the gene. The 3.6 kb <u>Rsa</u>I/<u>Eag</u>I vector fragment from
pSD456 was isolated and ligated with annealed synthetic
oligonucleotides MPSYN59 (SEQ ID NO:15), MPSY62 (SEQ ID
25   NO:16), MPSYN60 (SEQ ID NO:17), and MPSYN 61
(SEQ ID NO:18)

```
          RsaI
MPSYN59 5'
ACACGAATGATTTTCTAAAGTATTTGGAAAGTTTTATAGGTAGTTGATAGA-
30  MPSYN62 3'
TGTGCTTACTAAAAGATTTCATAAACCTTTCAAAATATCCATCAACTATCT   5'

MPSYN59    -ACAAAATACATAATTT 3'
```

35

```
BglII
MPSYN60 5'
TGTAAAAATAAATCACTTTTTATACTAAGATCT-
MPSYN61 3'
40  TGTTTTATGTATTAAAACATTTTTATTTAGTGAAAAATATGATTCTAGA-

          SmaI  PstI  EagI
MPSYN60    -CCCGGGCTGCAGC        3'
```

MPSYN61    -GGGCCCGACGTCGCCGG  5'

reconstructing the DNA sequences upstream from the A56R
ORF and replacing the A56R ORF with a polylinker region
5  as indicated above.  The resulting plasmid is pSD466.
The vaccinia deletion in pSD466 encompasses positions
[161,185-162,053].  The site of the deletion in pSD466 is
indicated by a triangle in FIG. 4.

         A 3.2 kb BglII/BamHI (partial) cassette
10 containing the E. coli Beta-galactosidase gene (Shapira
et al., 1983) under the control of the vaccinia 11 kDa
promoter (Bertholet et al., 1985; Guo et al., 1989) was
inserted into the BglII site of pSD466, forming
pSD466KBG.  Plasmid pSD466KBG was used in recombination
15 with rescuing virus vP618.  Recombinant vaccinia virus,
vP708, containing Beta-galactosidase in the A56R
deletion, was isolated as a blue plaque in the presence
of X-gal.

         Beta-galactosidase sequences were deleted from
20 vP708 using donor plasmid pSD467.  pSD467 is identical to
pSD466, except that EcoRI, SmaI and BamHI sites were
removed from the pUC/vaccinia junction by digestion of
pSD466 with EcoRI/BamHI followed by blunt ending with
Klenow fragment of E. coli polymerase and ligation.
25 Recombination between vP708 and pSD467 resulted in
recombinant vaccinia deletion mutant, vP723, which was
isolated as a clear plaque in the presence of X-gal.

    (5)  Construction of Plasmid pMPCSK1Δ for Deletion of
         Open Reading Frames [C7L-K1L]

30        Referring now to FIG. 5, the following vaccinia
clones were utilized in the construction of pMPCSK1Δ.
pSD420 is SalI H cloned into pUC8.  pSD435 is KpnI F
cloned into pUC18.  pSD435 was cut with SphI and
religated, forming pSD451.  In pSD451, DNA sequences to
35 the left of the SphI site (pos. 27,416) in HindIII M are
removed (Perkus et al., 1990).  pSD409 is HindIII M
cloned into pUC8.

25

        To provide a substrate for the deletion of the
[C7L-K1L] gene cluster from vaccinia, *E. coli* Beta-
galactosidase was first inserted into the vaccinia M2L
deletion locus (Guo et al., 1990) as follows.  To
5  eliminate the BglII site in pSD409, the plasmid was cut
with BglII in vaccinia sequences (pos. 28,212) and with
BamHI at the pUC/vaccinia junction, then ligated to form
plasmid pMP409B.  pMP409B was cut at the unique SphI site
(pos. 27,416).  M2L coding sequences were removed by
10 mutagenesis (Guo et al., 1990; Mandecki, 1986) using
synthetic oligonucleotide.

                                                        BglII
    MPSYN82 (SEQ ID NO:19) 5'
    TTTCTGTATATTTGCACCAATTTAGATCTTACTC
15                              AAAATATGTAACAATA 3'

    The resulting plasmid, pMP409D, contains a unique BglII
site inserted into the M2L deletion locus as indicated
above.  A 3.2 kb BamHI (partial)/BglII cassette
20 containing the *E. coli* Beta-galactosidase gene (Shapira
et al., 1983) under the control of the 11 kDa promoter
(Bertholet et al., 1985) was inserted into pMP409D cut
with BglII.  The resulting plasmid, pMP409DBG (Guo et
al., 1990), was used as donor plasmid for recombination
25 with rescuing vaccinia virus vP723.  Recombinant vaccinia
virus, vP784, containing Beta-galactosidase inserted into
the M2L deletion locus, was isolated as a blue plaque in
the presence of X-gal.
        A plasmid deleted for vaccinia genes [C7L-K1L]
30 was assembled in pUC8 cut with SmaI, HindIII and blunt
ended with Klenow fragment of *E. coli* polymerase.  The
left flanking arm consisting of vaccinia HindIII C
sequences was obtained by digestion of pSD420 with XbaI
(pos. 18,628) followed by blunt ending with Klenow
35 fragment of *E. coli* polymerase and digestion with BglII
(pos. 19,706).  The right flanking arm consisting of
vaccinia HindIII K sequences was obtained by digestion of
pSD451 with BglII (pos. 29,062) and EcoRV (pos. 29,778).
The resulting plasmid, pMP581CK is deleted for vaccinia

26

sequences between the BglII site (pos. 19,706) in HindIII
C and the BglII site (pos. 29,062) in HindIII K. The
site of the deletion of vaccinia sequences in plasmid
pMP581CK is indicated by a triangle in FIG. 5.

5              To remove excess DNA at the vaccinia deletion
junction, plasmid pMP581CK, was cut at the NcoI sites
within vaccinia sequences (pos. 18,811; 19,655), treated
with Bal-31 exonuclease and subjected to mutagenesis
(Mandecki, 1986) using synthetic oligonucleotide MPSYN233

10   (SEQ ID NO:20)
5' TGTCATTTAACACTATACTCATATTAATAAAAATAATATTTATT 3'.
The resulting plasmid, pMPCSK1Δ, is deleted for vaccinia
sequences positions 18,805-29,108, encompassing 12
vaccinia open reading frames [C7L - K1L]. Recombination

15   between pMPCSK1Δ and the Beta-galactosidase containing
vaccinia recombinant, vP784, resulted in vaccinia
deletion mutant, vP804, which was isolated as a clear
plaque in the presence of X-gal.

     (6)  **Construction of Plasmid pSD548 for Deletion of Large**

20        **Subunit, Ribonucleotide Reductase (I4L)**

             Referring now to FIG. 6, plasmid pSD405
contains vaccinia HindIII I (pos. 63,875-70,367) cloned
in pUC8. pSD405 was digested with EcoRV within vaccinia
sequences (pos. 67,933) and with SmaI at the pUC/vaccinia

25   junction, and ligated, forming plasmid pSD518. pSD518
was used as the source of all the vaccinia restriction
fragments used in the construction of pSD548.

             The vaccinia I4L gene extends from position
67,371-65,059. Direction of transcription for I4L is

30   indicated by an arrow in FIG. 6. To obtain a vector
plasmid fragment deleted for a portion of the I4L coding
sequences, pSD518 was digested with BamHI (pos. 65,381)
and HpaI (pos. 67,001) and blunt ended using Klenow
fragment of E. coli polymerase. This 4.8 kb vector

35   fragment was ligated with a 3.2 kb SmaI cassette
containing the E. coli Beta-galactosidase gene (Shapira
et al., 1983) under the control of the vaccinia 11 kDa

promoter (Bertholet et al., 1985; Perkus et al., 1990),
resulting in plasmid pSD524KBG.  pSD524KBG was used as
donor plasmid for recombination with vaccinia virus
vP804.  Recombinant vaccinia virus, vP855, containing
5   Beta-galactosidase in a partial deletion of the I4L gene,
was isolated as a blue plaque in the presence of X-gal.
        To delete Beta-galactosidase and the remainder
of the I4L ORF from vP855, deletion plasmid pSD548 was
constructed.  The left and right vaccinia flanking arms
10  were assembled separately in pUC8 as detailed below and
presented schematically in FIG. 6.
        To construct a vector plasmid to accept the
left vaccinia flanking arm, pUC8 was cut with <u>Bam</u>HI/<u>Eco</u>RI
and ligated with annealed synthetic oligonucleotides
15  518A1/518A2
    (SEQ ID NO:21/SEQ ID NO:22)
            <u>Bam</u>HI    <u>Rsa</u>I
    518A1   5'
    GATCCTGAGTACTTTGTAATATAATGATATATATTTTCACTTTATCTCAT
20  518A2   3'
    GACTCATGAAACATTATATTACTATATATAAAAGTGAAATAGAGTA


                    <u>Bgl</u>II    <u>Eco</u>RI
        TTGAGAATAAAAAGATCTTAGG        3'    518A1
25      AACTCTTATTTTTCTAGAATCCTTAA 5'      518A2


forming plasmid pSD531.  pSD531 was cut with <u>Rsa</u>I
(partial) and <u>Bam</u>HI and a 2.7 kb vector fragment
isolated.  pSD518 was cut with <u>Bgl</u>II (pos. 64,459)/ <u>Rsa</u>I
30  (pos. 64,994) and a 0.5 kb fragment isolated.  The two
fragments were ligated together, forming pSD537, which
contains the complete vaccinia flanking arm left of the
I4L coding sequences.
        To construct a vector plasmid to accept the
35  right vaccinia flanking arm, pUC8 was cut with
<u>Bam</u>HI/<u>Eco</u>RI and ligated with annealed synthetic
oligonucleotides 518B1/518B2
    (SEQ ID NO:23/SEQ ID NO:24)
            <u>Bam</u>HI <u>Bgl</u>II SmaI
40  518B1   5'
    GATCCAGATCTCCCGGGAAAAAAATTATTTAACTTTTCATTAATAGGGATTT

```
518B2     3'
GTCTAGAGGGCCCTTTTTTTTAATAAATTGAAAAGTAATTATCCCTAAA
```

```
                        RsaI    EcoRI
5            GACGTATGTAGCGTACTAGG      3'    518B1
             CTGCATACTACGCATGATCCTTAA  5'    518B2
```

forming plasmid pSD532. pSD532 was cut with RsaI
(partial)/EcoRI and a 2.7 kb vector fragment isolated.

10  pSD518 was cut with RsaI within vaccinia sequences (pos.
67,436) and EcoRI at the vaccinia/pUC junction, and a 0.6
kb fragment isolated. The two fragments were ligated
together, forming pSD538, which contains the complete
vaccinia flanking arm to the right of I4L coding

15  sequences.

        The right vaccinia flanking arm was isolated as
a 0.6 kb EcoRI/BglII fragment from pSD538 and ligated
into pSD537 vector plasmid cut with EcoRI/BglII. In the
resulting plasmid, pSD539, the I4L ORF (pos. 65,047-

20  67,386) is replaced by a polylinker region, which is
flanked by 0.6 kb vaccinia DNA to the left and 0.6 kb
vaccinia DNA to the right, all in a pUC background. The
site of deletion within vaccinia sequences is indicated
by a triangle in FIG. 6. To avoid possible recombination

25  of Beta-galactosidase sequences in the pUC-derived
portion of pSD539 with Beta-galactosidase sequences in
recombinant vaccinia virus vP855, the vaccinia I4L
deletion cassette was moved from pSD539 into pRC11, a pUC
derivative from which all Beta-galactosidase sequences

30  have been removed and replaced with a polylinker region
(Colinas et al., 1990). pSD539 was cut with EcoRI/PstI
and the 1.2 kb fragment isolated. This fragment was
ligated into pRC11 cut with EcoRI/PstI (2.35 kb), forming
pSD548. Recombination between pSD548 and the Beta-

35  galactosidase containing vaccinia recombinant, vP855,
resulted in vaccinia deletion mutant vP866, which was
isolated as a clear plaque in the presence of X-gal.

        DNA from recombinant vaccinia virus vP866 was
analyzed by restriction digests followed by

40  electrophoresis on an agarose gel. The restriction

patterns were as expected. Polymerase chain reactions
(PCR) (Engelke et al., 1988) using vP866 as template and
primers flanking the six deletion loci detailed above
produced DNA fragments of the expected sizes. Sequence

5    analysis of the PCR generated fragments around the areas
of the deletion junctions confirmed that the junctions
were as expected. Recombinant vaccinia virus vP866,
containing the six engineered deletions as described
above, was designated vaccinia vaccine strain "NYVAC."

10   **Example 2 - EXPRESSION OF HIV GENE PRODUCTS BY**
                  **HOST-RESTRICTED POXVIRUSES**

          This Example describes the generation of host-
restricted poxviruses that express HIV gene products.
The vectors employed are NYVAC, ALVAC and TROVAC.

15   **ALVAC and NYVAC Recombinants Containing the HIV-1 (IIIB)**
    **V3 Loop and Epitope 88**

          A 150 bp fragment encompassing the V3 loop
(amino acids 299-344; Javeherian et al., 1989; La Rosa et
al., 1990) of HIV-1 (IIIB) was derived by PCR using

20   oligonucleotides HIV3BL5 (SEQ ID NO:25) (5'-
ATGGTAGAAATTAATTGTAC-3') and HIV3BL3 (SEQ ID NO:26) (5'-
ATCATCGAATTCAAGCTTATTATTTTGCTCTACTAATGTTAC-3') with
pHXB.2D (III) as template (provided by Dr. R.C. Gallo,
NCI-NIH, Bethesda, MD). Oligonucleotides HIV88A (SEQ ID

25   NO:27) (5'-
ATGAATGTGACAGAAAATTTTAACATGTGGAAAAATGTAGAAATTAATTGTACAAGA
CCC-3') and HIV88B (SEQ ID NO:28) (5'-
GGGTCTTGTACAATTAATTTCTACATTTTTCCACATGTTAAAATTTTCTGTCACATT
CAT-3') were annealed together to produce a double-

30   stranded fragment containing the HIV-1 epitope 88 (amino
acids 95-105, Shaffermann et al., 1989). The 150 bp V3-
containing PCR fragment containing the epitope and the 42
bp fragment containing the 88 epitope sequences were
fused together by PCR by virtue of the existence of

35   complementary sequences. The reactions were performed
using oligonucleotides HIV88C (SEQ ID NO:29) (5'-
AGTAATGTGACAGAAAATTTTAAC-3') and HIV3BL3. The 192 bp

PCR-derived fragment contains the epitope 88 sequences
fused upstream to the V3 loop sequences. A termination
codon (TAA) was incorporated into oligonucleotide
HIV3BL3P to terminate translation of the open reading

5  frame and an initiation codon was incorporated into
oligonucleotide HIV88C to serve as the start of
translation to express the epitope 88/V3 loop fusion
protein. Additionally, oligonucleotide HIV3BL3 was
synthesized so that an EcoRI site existed at the 3'-end

10 of the 192 bp PCR fragment.

The entomopoxvirus promoter, 42 kDa (early) was
generated by PCR using oligonucleotides RG273 (SEQ ID
NO:30) (5'-AGGCAAGCTTTCAAAAAAATATAAATGATTC-3') and RG274
(SEQ ID NO:31) (5'-TTTATATTGTAATTATATATTTTC-3') with

15 plasmid, pAM12, as template. The 108 bp fragment
containing the 42 kDa promoter was synthesized to contain
a HindIII site at the 5'-end. The 42 kDa promoter
containing segment was kinased and digested with HindIII
prior to ligation to the epitope 88/V3 fragment digested

20 with EcoRI and pRW831 digested with HindIII and EcoRI.
The resultant plasmid was designated as pC5HIVL88. This
plasmid was used in in vitro recombination assays with
CPpp as rescue virus to generate vCP95. ALVAC
recombinant, vCP95, contains the epitope 88/V3 loop in

25 the de-ORFed C5 locus of CPpp.

The plasmid pC5HIVL88 was digested with HindIII
and EcoRI to liberate a 300 bp fragment containing the
epitope 88/V3 expression cassette described above. This
fragment was excised from a LMP-agarose gel and isolated

30 by phenol extraction (2X) and ether extraction (1X). The
isolated fragment was blunt-ended using the Klenow
fragment of the E. coli DNA polymerase in the presence of
2mM dNTPs. The blunted fragment was ligated to pSD550VC
digested with SmaI to yield plasmid pHIVL88VC. This

35 plasmid was used with vP866 as the rescue virus to
generate vP878. vP878 contains the epitope 88/V3 loop
cassette in the de-ORFed I4L locus of vP866.

## ALVAC- and NYVAC-Based Recombinants Expressing the HIV-1
## (IIIB) Envelope Glycoproteins

An expression cassette composed of the HIV-1
(IIIB) env gene juxtaposed 3' to the vaccinia virus H6
5   promoter (Guo et al., 1989; Taylor et al., 1988a,b) was
engineered for expression of gp160 from HIV-1 by the
ALVAC and NYVAC vectors. A 1.4 kb fragment was amplified
from pHXB.2D (III) (provided by Dr. R.C. Gallo, NCI-NIH,
Bethesda, MD) using oligonucleotides HIV3B1 (SEQ ID
10  NO:32) (5'-GTTTTAATTGTGGAGGGGAATTCTTCTACTGTAATTC-3') and
HIV3B5 (SEQ ID NO:33) (5'-
ATCATCTCTAGAATAAAAATTATAGCAAAATCCTTTC-3'). This fragment
contains the 3' portion of the env gene. PCR
amplification with these primers placed a vaccinia virus
15  early transcription termination T5NT sequence motif
following the coding sequence and removed the T5NT motif
situated at position 6146 to 6152 (Ratner et al., 1985)
without altering the amino acid sequence. This change (T
to C) creates an EcoRI site (GAATTC) at this position.
20  This 1.4 kb fragment was digested with EcoRI (5'-end) and
XbaI (3'- end) and inserted into EcoRI and XbaI digested
pBS-SK (Stratagene, La Jolla, CA). The resultant plasmid
was designated as pBSHIVENV1,5. Nucleotide sequence
analysis of this fragment demonstrated that the sequence
25  was entirely correct except for a T to C transition at
position 7048. This transition was corrected as follows:
A 250 bp fragment was derived by PCR using
oligonucleotides HIV3B1 (SEQ ID NO:32) (5'-
GTTTTAATTGTGGAGGGGAATTCTTCTACTGTAATTC-3') and HIV3B17
30  (SEQ ID NO:34) (5'-TGCTACTCCTAATGGTTC-3') with pHXB.2D
(III) as template. This fragment was digested with BglII
and EcoRI. The fragment was inserted into pBSHIV3B1,5,
digested with BglII and EcoRI and thus substituted for
the region with the incorrect nucleotide to yield plasmid
35  pBSHIV3B3P.

PCR was utilized to derive a 150 bp fragment
containing the 5' portion of the env gene with

32

oligonucleotides HIV3B9 (SEQ ID NO:35) (5'-
CATATGCTTTAGCATCTGATG-3') and HIV3B10 (SEQ ID NO:36) (5'-
ATGAAAGAGCAGAAGACAGTG-3') with pHXB.2D (III) as template.
PCR was also used to generate a 128 bp fragment
5   containing the vaccinia virus H6 promoter from pC3FGAG
using oligonucleotides VV6K5P (SEQ ID NO:37) (5'-
ATCATCGGTACCGATTCTTTATTCTATAC-3') and VVH63P (SEQ ID
NO:38) (5'-TACGATACAAACTTAACGG-3'). Both fragments were
digested with KpnI and the 150 bp fragment was kinased
10  prior to co-insertion of these fragments into pBS-SK
digested with KpnI. The resultant plasmid was designated
as pBSH6HIV3B5P.

        PCR was used to generate a 600 bp fragment from
pHXB.2D (III) with oligonucleotides HIV3B2 (SEQ ID NO:39)
15  (5'-GAATTACAGTAGAAGAATTCCCCTCCACAATTAAAAC-3') and HIV3B7
(SEQ ID NO:40) (5'-CAATAGATAATGATACTAC-3'). This
fragment was digested with EcoRI and kinased. PCR was
also used to derive a 500 bp fragment with the same
template but with oligonucleotides HIV3B6 (SEQ ID NO:41)
20  (5'-GTATTATATCAAGTTTATATAATAATGCATATTC-3') and HIV3B8
(SEQ ID NO:42) (5'-GTTGATGATCTGTAGTGC-3'). This fragment
was digested with KpnI. These fragments together
correspond to nucleotide 5878 to 6368 (Ratner et al.,
1985). The engineering of these fragments with these
25  primers also removes a T5NT sequence positioned at
nucleotide 6322 to 6328 without altering the amino acid
sequence. These two fragments were inserted into
pBSHIV3B3P digested with KpnI and EcoRI. This plasmid
was designated as pBSHIV3BP2768.

30      Plasmid pBSH6HIV3B5P was digested with KpnI to
liberate a 360 bp fragment containing the H6 promoter and
the 5' portion (150 bp) of the HIV-1 env gene. This KpnI
fragment was ligated into pBSHIV3B3P2768 digested with
KpnI to yield plasmid pBSHIV3BEII. A 2.8 kb fragment was
35  derived from pBSHIV3BEII by digestion with XbaI followed
by a partial KpnI digestion. This fragment was blunt-
ended and inserted into SmaI digested pSD550. The

plasmid pI4LH6HIV3B was generated and used in
recombination experiments with vP866 as the rescue virus.
This generated vP911 which contains the HIV-1 env gene in
the I4L locus of the NYVAC genome.

5            To insert the HIV-1 env gene into an ALVAC
vector, pBSHIV3BEAII was digested with NruI and XbaI.
The derived 2.7 kb fragment was blunt-ended with the
Klenow fragment of the *E. coli* DNA polymerase in the
presence of 2mM dNTPs.  This fragment contains the entire
10  HIV-1 env gene juxtaposed 3' to the 3'-most 21 bp (to
NruI site) of the vaccinia H6 promoter.  This fragment
was ligated to a 3.1 kb fragment derived by digestion of
pRW838 with NruI and EcoRI with subsequent blunt-ending
with Klenow.  The pRW838 derived fragment contains the
15  homologous arms derived from canarypox to direct the
foreign gene to the C5 locus.  It also contains the 5'-
most 100 bp of the H6 promoter.  Therefore, ligation of
these fragments resulted in an insertion plasmid
containing an expression cassette for the HIV-1 env gene
20  and was designated pC5HIV3BE.  This plasmid was used in
*in vitro* recombination experiments with ALVAC as the
rescue virus to generate vCP112.
**NYVAC-Based Recombinants Expressing the HIV-1 (IIIB)**
**gp120**
25           The plasmid pBSHIV3BEAII was digested with
EcoRI and XbaI to liberate a 4.3 kb fragment.  This
fragment contains the vaccinia virus H6 promoter linked
to the HIV-1 env gene to nucleotide 6946 (Ratner et al.,
1985).  The 4.3 kb fragment was ligated to 300 bp
30  EcoRI/XbaI digested PCR-derived fragment corresponding to
the 3' portion of the gp120 coding sequence.  The 300 bp
PCR fragment was derived using oligonucleotides HIV1-120A
(SEQ ID NO:43) (5'-
ATCATCTCTAGAATAAAAATTATGGTTCAATTTTTACTACTTTTATATTATATATTT
35  C-3') and HIV1-120B (SEQ ID NO:44) (5'-
CAATAATCTTTAAGCAAATCCTC-3') with pHXB.2D (III) as
template.  The ligation of the 4.3 kb XbaI/EcoRI fragment

34

and the 300 bp XbaI/EcoRI fragment yielded plasmid
pBSHIVB120.

A 1.6 kb KpnI/XbaI fragment was derived from
pBSHIVB120 by initially linearizing the plasmid with XbaI
5    followed by a partial KpnI digestion.  The 1.6 kb
fragment was blunt-ended by treatment with the Klenow
fragment of the *E. coli* DNA polymerase in the presence of
2mM dNTPs.  This fragment was inserted into pSD541
digested with SmaI to yield pATIHIVB120.  This plasmid
10   was used in *in vitro* recombination experiments to
generate vP921.  This recombinant contains the portion of
the HIV-1 env gene encoding gp120 in the ATI locus of
NYVAC.

To determine the authenticity of the HIV-1 gene
15   products expressed by vP911, vP921 and vCP112,
immunoprecipitation analyses were performed.

Lysates derived from the infected cells were
analyzed for HIV-1 env gene expression using pooled serum
from HIV-1 seropositive individuals (obtained from Dr.
20   Genoveffa Franchini, NCI-NIH, Bethesda, MD).  The sera
was preadsorbed with vP866-infected Vero cells.  The
preadsorbed human sera was bound to protein A-sepharose
in an overnight inoculation at 4°C.  In some cases a
monoclonal antiserum specific to gp120 (Dupont) was used
25   as the primary serum and a rat anti-mouse as the second
antibody.  Following this incubation period, the material
was washed 4 times with 1X Buffer A.  Lysates precleared
with normal human sera and protein A-Sepharose were then
incubated overnight at 4°C with the human sera from
30   seropositive individuals bound to protein A-Sepharose.
Following the overnight incubation period, the samples
were washed four times with 1X Buffer A and 2X with
LiCl$_2$/urea buffer.  Precipitated proteins were
dissociated from the immune complexes by the addition of
35   2X Laemmli's buffer (125mM Tris (pH6.8), 4% SDS, 20%
glycerol; 10% 2-mercaptoethanol) and boiling for 5
minutes.  Proteins were fractionated on a 10% Dreyfuss

35

gel system (Dreyfuss et al., 1984) fixed and treated with
1M - Na - salicylate for fluorography.

        The results of immunoprecipitation using sera
pooled from HIV-1 seropositive individuals showed
5   specific precipitation of the gp120 and gp41 mature forms
of the gp160 envelope glycoprotein from vP911 infected
cell lysates. No such specific gene products can be
detected in the parental virus (NYVAC; vP866) infected
cell lysates. Specific precipitation of gp120 was also
10  found in vP921 infected cell lysates.

        Immunofluorescence analysis with the same sera
illustrated that the gp160 and gp120 species expressed by
vP911 and vP921, respectively, were present on the
surface of infected cells.

15      Immunoprecipitation was also performed with
vCP112 infected CEF cells. No HIV-specific polypeptides
were precipitated with a monoclonal antibody directed
against the gp120 extracellular moiety from cells
infected with the ALVAC parental virus and uninfected CEF
20  cells. Two HIV-specific polypeptides species were,
however, precipitated from vCP112 infected cells. These
species migrated with apparent mobilities of 160 kDa and
120 kDa, corresponding to the precursor _env_ gene product
and the mature extracellular form, respectively.

25  **A Recombinant Vaccinia Virus Expressing HIV gp120 Elicits**
    **Primary HIV-specific Cytotoxic T Lymphocyte Activity**

        Following iv administration with $5 \times 10^7$ PFUs of
vaccinia virus recombinants vP878, vP911, or vP921, or,
as a control, with NYVAC, the vector, splenic CTL
30  activity of BALB/c mice was assessed against syngeneic
P815 cells which had been incubated overnight with
peptide HBX2 (Table 1). ɔdest, but significant ($P < 0.05$)
primary CTL activity was generated in the spleens of mice
administered vP921, expressing HIV gp120. No other
35  recombinant vaccinia virus nor the vector was able to
elicit primary HIV-specific CTL activity. This was not
due to inadequate infection as each group of mice

36

administered a vaccinia virus responded with primary
vaccinia-specific CTL activity.  Control, unimmunized
mice responded to neither target.

**Recombinant Poxviruses Expressing HIV env Peptides**
5  **Generate HIV-Specific Memory Cytotoxic T Lymphocytes**

At least one month following a single
inoculation with one of the recombinant vaccinia viruses,
mouse spleen cells were stimulated *in vitro* with
syngeneic, naive spleen cells previously infected with
10  NYVAC or with each of the HIV recombinant vaccinia
viruses (Table 2).  Strong HIV-specific CTL activity was
detected only in the spleen cell cultures of mice
immunized with vP878, vP911, and vP921 which were
restimulated *in vitro* by cells infected with one of the
15  same vaccinia virus HIV recombinants (vP878, vP911, or
vP921).  The vaccinia virus recombinants expressing HIV
gp120 or gp160 were better able to generate memory CTLs
than the vaccinia virus recombinant expressing only the
V3 loop fused to the 88 epitope.  HIV-specific memory CTL
20  activity could not be elicited from unimmunized control
or NYVAC immunized spleen cells.  The absence of HIV-
specific CTL activity from vector immunized mice could
not be attributed to poor immunization since vaccinia-
specific memory CTL activity was apparent after *in vitro*
25  stimulation with spleen cells infected with any of the
vaccinia viruses used.

In a similar study, the ability of a canarypox
recombinant expressing the V3 loop region fused to the 88
epitope (vCP95) to generate HIV-specific memory CTLs was
30  examined (Table 3).  Three weeks following a single
inoculation of $10^8$ PFUs of vCP95 or the canarypox vector,
CPpp, HIV-specific memory CTL responses were compared to
that elicited by the recombinant vaccinia virus analog,
vP878.  Vaccinia and canarypox CTL responses were
35  included as controls for proper immunization.  Only
spleen cells from vP878 and vCP95 immunized mice produced
HIV-specific memory CTL activity which could be

stimulated by vP878. The inability of vCP95 to stimulate
existing memory CTLs to functional cytolytic CTLs may
have been related to the *in vitro* conditions employed
which were maximized based upon the use of vaccinia virus
5 recombinants. Nonetheless, vCP95 was fully capable of
generating significant HIV-specific memory CTLs in the
spleens of immunized mice.

**Characterization of the *In Vitro* Stimulated Cytotoxic
Cells**

10          It is conceivable that the cells mediating
cytotoxicity against the HIV peptide-pulsed target cells
represent a population of nonspecific effector cells
unrelated to CTLs, such as natural killer cells. To test
this, the spleen cells of mice immunized with vP921 and
15 restimulated *in vitro* with vP921 infected spleen cells
were depleted of T-lymphocytes bearing surface antigens
characteristic of T helper lymphocytes (CD4) or of
cytotoxic T lymphocytes (CD8) and assayed against V3 loop
peptide pulsed target cells (Table 4). As before, only
20 vP921 immunized mice generated memory HIV-specific CTL
activity which could be stimulated *in vitro* with v 21
infected syngeneic spleen cells. Although the complement
preparation (C') and the monoclonal anti-CD4 and anti-CD8
produced some toxic effects, only the cultures depleted
25 of CD8-bearing cells (anti-CD8 + C') were also depleted
of HIV-specific cytotoxic effector cells. Thus, the
cells mediating cytolytic activity against the HIV
peptide-pulsed target cells possessed CD8 antigens on
their cell membranes, a characteristic of MHC class I
30 restricted CTLs.

**Specificity of CTL Antigen Receptor Recognition of the V3
Loop Region of HIV gp120**

          T lymphocyte antigen receptors are exquisitely
sensitive to small alterations in the primary amino acid
35 sequence of the epitope fragment. The V3 loop region of
HIV gp120 is hypervariable and differs immu. ologically
among HIV isolates. The hypervariability resides in

38

substitutions and additions of only a few amino acids. To examine the specificity of cytotoxic cells generated by HIV vaccinia virus recombinants, susceptibility to CTL activity was compared among P815 target cells pulsed with

5 peptides corresponding the V3 loop region of HIV isolates $III_B$, SF2, and MN. Only immunization with vP911 and vP921 induced HIV specific primary CTL activity (Table 5). Furthermore, HIV specific CTL activity was confined only to P815 target cells pulsed with peptide

10 corresponding to the V3 loop of HIV isolate $III_B$. Similar results were obtained with in vitro stimulated, HIV specific secondary CTL activity induced by immunization with the vaccinia virus recombinants vP878, vP911, and vP921 (Table 6). Thus, HIV specific CTLs

15 elicited by recombinant vaccinia viruses expressing various portions of the <u>env</u> gene of HIV isolate $III_B$ recognize only target epitopes derived from the same antigenic isolate.

<u>Lymphocyte Proliferation Responses to HIV Epitopes</u>
20 <u>Following Immunization with Vaccinia Virus HIV</u>
<u>Recombinants</u>

Lymphocyte proliferation to antigens is an in vitro correlate of cell-mediated immunity. Presentation of the appropriate antigen induces cellular proliferation

25 in the immune population of cells expressing receptors for the antigen. The initiation and continuation of proliferation requires the involvement of T helper lymphocytes via soluble mediators. To evaluate cell-mediated immunity to HIV antigens in mice immunized with

30 recombinant vaccinia viruses expressing HIV antigens, spleen cells from mice immunized 27 days earlier were incubated for 5 days with peptides correlating to T helper lymphocyte epitopes designated $T_1$ and $T_2$, as well as with purified HIV gp160 (Table 7). No proliferative

35 responses to the T helper cell epitopes $T_1$ and $T_2$ were observed in any of the spleen cell cultures. However, the spleen cells of mice previously immunized with vP921

39

vigorously responded to HIV gp160 as determined by the
incorporation of [$^3$H]-thymidine. A stimulation index
(SI) of greater than 2.0 is considered indicative of
immunity. Thus, inoculation of mice with vP921 elicited
5 cell-mediated immunity to HIV gp160.

**Antibody Responses of Mice Inoculated with Vaccinia Virus**
**HIV Recombinants**

To evaluate humoral responses to HIV, mice were
immunized at day 0 with one of the vaccinia virus HIV
10 recombinants and received a secondary immunization at
week 5. The mice were bled at various intervals through
9 weeks after the initial immunization. Pooled sera from
each treatment group were assayed for antibodies to HIV
by ELISA employing purified gp160 as antigen (Table 8).
15 Primary antibody responses were generally modest, but
detectable with the highest levels induced by vP911.
Following the secondary immunization, the antibody titers
of mice immunized with vP911 and vP921 increased and
peaked at week 7 with titers of over 4,600 and 3,200,
20 respectively, before declining slightly by week 9. Thus,
two vaccinia virus HIV recombinants, vP911 and vP921,
were capable of inducing a significant antibody response.

40

Table 1.   Primary CTL activity of spleen cells from
           mice immunized with vaccinia virus
           recombinants against vaccinia virus
           infected targets and targets pulsed with
5          peptide corresponding to the V3 loop
           region of HIV-1 gp120.

| | PERCENT CYTOTOXICITY | | |
| | TARGET | | |
| IMMUNIZATION | P815 | VAC | HIV V3 |
|---|---|---|---|
| NONE | −3.5 ± 2.0 | −0.6 1.5 | −4.8 1.6 |
| NYVAC | −4.4 ± 1.9 | 9.5 * 3.2 | −5.9 1.7 |
| vP878 | −4.9 ± 1.8 | 7.1 * 2.2 | −4.0 1.2 |
| vP911 | −4.0 ± 2.5 | 4.6 * 2.0 | 1.4 5.1 |
| vP921 | −3.4 ± 0.9 | 10.7 * 1.5 | 15.5 * 2.8 |

E:T = 100:1

* P<0.05 vs appropriate controls, Student's t-test

Table 2.  Secondary CTL activity of spleen cells
following *in vitro* stimulation with
vaccinia virus recombinants.

| IMMUNIZATION | | PERCENT CYTOTOXICITY TARGET | | |
| --- | --- | --- | --- | --- |
| *in vivo* | *in vitro* | P815 | VAC | HIV V3 |
| NONE | NONE | -0.1 | 1.9 | 0.5 |
|  | NYVAC | 3.7 | 8.9 | 3.8 |
|  | vP878 | 4.6 | 9.0 | 5.5 |
|  | vP911 | -1.7 | 2.9 | 4.8 |
|  | vP921 | 2.9 | 2.9 | 1.5 |
| NYVAC | NONE | 0.0 | 4.4 | 1.1 |
|  | NYVAC | 3.5 | 47.8 * | 9.2 |
|  | vP878 | 6.3 | 44.1 * | 14.4 |
|  | vP911 | 7.9 | 48.6 * | 10.6 |
|  | vP921 | 6.8 | 50.8 * | 7.9 |
| vP878 | NONE | 0.1 | 1.7 | 1.3 |
|  | NYVAC | 10.2 | 58.5 * | 13.0 |
|  | vP878 | 11.6 | 57.9 * | 59.9 * |
|  | vP911 | 7.8 | 56.2 * | 40.8 * |
|  | vP921 | 4.9 | 42.0 * | 14.8 |
| vP911 | NONE | 0.3 | 2.9 | 4.0 |
|  | NYVAC | 6.2 | 50.7 * | 8.5 |
|  | vP878 | 5.9 | 50.9 * | 77.4 * |
|  | vP911 | 5.0 | 54.2 * | 82.6 * |
|  | vP921 | 10.9 | 55.0 * | 87.8 * |
| vP921 | NONE | 2.9 | 5.0 | 9.4 |
|  | NYVAC | 8.3 | 54.4 * | 22.7 |
|  | vP878 | 10.4 | 56.2 * | 85.6 * |
|  | vP911 | 8.7 | 58.2 * | 86.5 * |
|  | vP921 | 7.8 | 55.2 * | 81.0 * |

BALB/cJ spleen cells from mice immunized approximately 1 month
earlier with the indicated vaccinia virus recombinants and were
incubated with infected syngeneic spleen cells for 5 days and
assayed for cytotoxicity at an effector to target cell ratio of
20:1.

* $P<0.05$ compared to controls, Student's *t*-test.

42

Table 3.  Anamnestic CTL responses of the spleen cells of mice administered a single inoculation of recombinant vaccinia or canarypox virus expressing the V3 loop of HIV gp120.

| IMMUNIZATION | | PERCENT CYTOTOXICITY | | | |
| --- | --- | --- | --- | --- | --- |
| | | TARGET | | | |
| PRIMARY *in vivo* | BOOSTER *in vitro* | P815 | Vac | CP | HIV V3 |
| NONE | NONE | 0.4 | −2.5 | −2.3 | −1.5 |
| | vP804 | 0.5 | 8.8 | 0.7 | 0.8 |
| | vP878 | 1.8 | 6.1 | 0.4 | 1.6 |
| | CP | 5.8 | 4.2 | 4.9 | 0.4 |
| | vCP95 | 4.4 | 2.6 | 6.1 | 0.1 |
| | SB135 | −0.2 | −0.7 | −0.4 | 0.5 |
| vP804 | NONE | 0.7 | 1.7 | 0.1 | 1.3 |
| | vP804 | 5.5 | 43.5 * | 5.8 | 3.5 |
| | vP878 | 3.6 | 42.5 * | 1.6 | −0.3 |
| | CP | 8.5 | 7.0 | 5.6 | 3.9 |
| | vCP95 | 5.8 | 5.3 | 4.4 | 4.0 |
| | SB135 | 1.2 | −0.9 | −0.5 | −0.2 |
| vP878 | NONE | 0.2 | −2.9 | −0.8 | −0.2 |
| | vP804 | 5.3 | 56.4 * | 7.5 | 4.1 |
| | vP878 | 6.7 | 60.2 * | 7.7 | 41.7 * |
| | CP | 8.7 | 13.4 | 9.4 | 4.7 |
| | vCP95 | 7.1 | 10.5 | 8.7 | 19.0 |
| | SB135 | 1.9 | −0.7 | −0.2 | −1.4 |
| CP | NONE | 4.6 | −0.6 | 2.3 | −0.0 |
| | vP804 | 11.0 | 17.7 * | 5.7 | 6.1 |
| | vP878 | 7.1 | 14.6 * | 12.3 | 5.5 |
| | CP | 7.4 | 5.9 | 19.3 * | 3.1 |
| | vCP95 | 6.8 | 5.4 | 20.4 * | 2.8 |
| | SB135 | 1.4 | −0.4 | 0.8 | −1.4 |
| vCP95 | NONE | −0.8 | −2.2 | −1.3 | 0.3 |
| | vP804 | 9.4 | 26.4 * | 9.3 | 6.6 |
| | vP878 | 10.4 | 22.5 * | 16.9 | 32.1 * |
| | CP | 8.8 | 7.2 | 20.0 * | 3.2 |
| | vCP95 | 5.1 | 4.2 | 19.6 * | 7.8 |
| | SB135 | 1.9 | −1.5 | −0.3 | −1.2 |

Twenty-three days after immunization, the spleen cells were stimulated *in vitro* for 5 days with virus infected or peptide-pulsed syngeneic spleen cells and then assayed for specific cytotoxicity against virus infected or peptide-pulsed P815 target cells at an effector to target cell ratio of 20:1. * $P < 0.05$ compared to appropriate controls, Student's *t*-test.

43

Table 4.  Depletion of cytotoxic activity with monoclonal antibodies to CD8 plus complement.

| IMMUNIZATION | | TREATMENT | PERCENT CYTOTOXICITY | | |
|---|---|---|---|---|---|
| | | | TARGETS | | |
| *in vivo* | *in vitro* | | P815 | VAC | HIV V3 |
| NONE | NONE | NONE | 1.1 | 1.5 | -0.3 |
| NONE | NYVAC | NONE | -7.4 | 0.4 | -0.4 |
| NONE | vP921 | NONE | -0.2 | 1.1 | -0.7 |
| NYVAC | NONE | NONE | -3.1 | -0.3 | -1.4 |
| NYVAC | NYVAC | NONE | -2.6 | 40.5 | -0.3 |
| NYVAC | vP921 | NONE | 3.3 | 31.4 | -2.9 |
| vP921 | NONE | NONE | 3.0 | -1.3 | -0.1 |
| vP921 | NYVAC | NONE | -4.9 | 25.9 | 12.2 |
| vP921 | vP921 | NONE | -0.2 | 21.3 | 30.5 |
| vP921 | vP921 | C' | 4.6 | 20.1 | 22.9 |
| vP921 | vP921 | anti-CD4 | 4.2 | 22.6 | 23.2 |
| vP921 | vP921 | anti-CD8 | -5.0 | 22.5 | 26.9 |
| vP921 | vP921 | anti-CD4+C' | 10.0 | 26.6 | 30.1 |
| vP921 | vP921 | anti-CD8+C' | 9.2 | 7.1 | 2.3 |

44

Table 5.   Specificity of primary CTL activity for
           the V3 loop of HIV-1 isolate III$_B$
           following a single inoculation with HIV
           recombinant vaccinia viruses.

| IMMUNIZATION | | PERCENT CYTOTOXICITY | | | |
|---|---|---|---|---|---|
| | | | TARGET | | |
| | | | V3 PEPTIDE | | |
| | | P815 | IIIB | SF2 | MN |
| NONE | | -2.7 | -1.9 | -0.9 | -1.2 |
| | ± | 0.5 | 0.5 | 0.5 | 0.5 |
| NYVAC | | -1.6 | -0.3 | -0.6 | -0.3 |
| | ± | 0.5 | 0.8 | 0.7 | 0.2 |
| vP878 | | -2.8 | 0.5 | -0.5 | -1.2 |
| | ± | 0.8 | 1.0 | 0.6 | 0.5 |
| vP911 | | -2.6 | 7.5 ° | -0.5 | -1.1 |
| | ± | 0.2 | 3.2 | 0.5 | 0.4 |
| vP921 | | -2.5 | 12.5 ° | -0.1 | -1.2 |
| | ± | 0.7 | 3.6 | 0.5 | 0.5 |

Mice were administered a single iv inoculation with
the indicated vaccinia virus recombinant and assayed
for CTL activity 7 days later against P815 targets
and P815 targets pulsed with one of three peptides
corresponding to the V3 loop region of HIV-1 isolates
III$_B$, SF2, and MN.   Although assayed at effector to
target cell ratios of 100:1, 50:1, and 25:1, only the
100:1 data are shown.

* $P < 0.05$ vs appropriate controls, Student's $t$-test

45

Table 6. Specificity of secondary CTL activity for the V3 loop of HIV-1 isolate III$_B$ following a single inoculation with HIV recombinant vaccinia viruses.

| IMMUNIZATION | | PERCENT CYTOTOXICITY TARGET | | | |
| --- | --- | --- | --- | --- | --- |
| | | | V3 PEPTIDE | | |
| in vivo | in vitro | P815 | IIIB | SF2 | MN |
| NONE | NONE | 1.0 | 1.1 | 0.5 | -0.0 |
| | NYVAC | 0.4 | 0.5 | -0.6 | -0.3 |
| | vP878 | 0.2 | 0.2 | -0.5 | -1.0 |
| | vP911 | -1.5 | 0.3 | -0.5 | 0.2 |
| | vP921 | -0.6 | 1.4 | 0.1 | -0.5 |
| NYVAC | NONE | -2.2 | 0.2 | 0.5 | -1.0 |
| | NYVAC | 3.2 | 2.2 | 3.9 | 2.5 |
| | vP878 | 4.4 | 5.9 | 5.0 | 6.1 |
| | vP911 | 5.8 | 11.1 | 5.0 | 5.3 |
| | vP921 | 5.0 | 6.5 | 2.9 | 2.9 |
| vP878 | NONE | 0.1 | -0.2 | -0.9 | -1.0 |
| | NYVAC | 3.0 | 4.8 | 4.4 | 4.5 |
| | vP878 | 7.9 | 20.2 | 7.8 | 8.6 |
| | vP911 | 4.8 | 7.8 | 4.5 | 4.7 |
| | vP921 | 2.7 | 6.9 | 2.8 | 3.0 |
| vP911 | NONE | 0.9 | 1.8 | 1.4 | 0.5 |
| | NYVAC | 8.8 | 8.3 | 8.1 | 6.6 |
| | vP878 | 6.6 | 57.2 | 6.8 | 8.2 |
| | vP911 | 4.6 | 63.7 | 2.9 | 4.2 |
| | vP921 | 7.2 | 63.6 | 4.1 | 4.9 |
| vP921 | NONE | 0.5 | 0.8 | 1.2 | 0.6 |
| | NYVAC | 4.4 | 7.9 | 7.5 | 6.0 |
| | vP878 | 8.1 | 59.0 | 7.1 | 7.5 |
| | vP911 | 6.4 | 71.4 | 7.9 | 6.6 |
| | vP921 | 9.3 | 63.4 | 9.0 | 8.1 |

Table 7.    Lymphocyte proliferative responses to HIV gp160 epitopes 27 days after a single immunization with HIV recombinant vaccinia viruses.

| | | | COUNTS PER MINUTE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | HIV ANTIGEN | | | | | | | | |
| | | | gp160 (μg) | | T1 (μg) | | | T2 (μg) | | | |
| IMMUNIZATION | | RPMI | 1 | 10 | 1 | 5 | 10 | 1 | 5 | 10 | |
| NONE | MEAN | 5,185 | 6,397 | 7,808 | 7,682 | 8,614 | 11,541 | 6,141 | 8,835 | 6,774 | |
| | ±SD | 1,020 | 2,174 | 2,596 | 1,274 | 2,033 | 2,036 | 2,103 | 1,883 | 2,806 | |
| | SI | 1.0 | 1.2 | 1.5 | 1.0 | 1.1 | 1.5 | 1.0 | 1.4 | 1.1 | |
| NYVAC | MEAN | 10,327 | 13,589 | 15,969 | 11,360 | 12,654 | 15,369 | 10,339 | 9,834 | 8,868 | |
| | ±SD | 1,543 | 3,323 | 4,583 | 1,352 | 2,272 | 1,821 | 762 | 1,731 | 502 | |
| | SI | 1.0 | 1.3 | 1.5 | 1.0 | 1.1 | 1.4 | 1.0 | 1.0 | 0.9 | |
| vP878 | MEAN | 10,126 | 13,150 | 18,329 | 11,114 | 11,956 | 13,754 | 10,415 | 11,442 | 9,147 | |
| | ±SD | 1,269 | 1,103 | 4,245 | 1,217 | 1,106 | 1,568 | 335 | 1,288 | 1,033 | |
| | SI | 1.0 | 1.3 | 1.8 | 1.0 | 1.1 | 1.2 | 1.0 | 1.1 | 0.9 | |
| vP911 | MEAN | 12,155 | 15,564 | 26,083 | 12,417 | 15,380 | 17,007 | 10,681 | 11,412 | 10,702 | |
| | ±SD | 1,307 | 9,707 | 16,327 | 873 | 1,847 | 6,266 | 2,428 | 3,201 | 1,468 | |
| | SI | 1.0 | 1.3 | 2.1 | 1.0 | 1.2 | 1.4 | 1.0 | 1.1 | 1.0 | |
| vP921 | MEAN | 9,701 | 49,256* | 61,036* | 10,550 | 15,367 | 15,816 | 8,818 | 9,232 | 8,803 | |
| | ±SD | 2,601 | 23,673 | 25,866 | 3,447 | 3,481 | 7,176 | 954 | 2,265 | 2,860 | |
| | SI | 1.0 | 5.1 | 6.3 | 1.0 | 1.5 | 1.5 | 1.0 | 1.0 | 1.0 | |

SI - stimulation index.

* $P<0.05$ compared to unstimulated control cultures

Student's t-test

47

Table 8.  HIV gp160 ELISA titers of mice immunized with HIV recombinant vaccinia viruses.

| IMMUNIZATION | WEEKS AFTER IMMUNIZATION | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 7 | 9 | |
| CONTROLS | 22 | 32 | 32 | 38 | 36 | 33 | |
| NYVAC | 38 | 36 | 37 | 28 | 50 | 45 | |
| vP878 | 20 | 43 | 27 | 46 | 65 | 63 | |
| vP911 | 0 | 0 | 90 | 453 | 4,614 | 3,263 | |
| vP921 | 0 | 26 | 25 | 77 | 2,614 | 1,689 | |

5

10

15

48

<u>Example 3</u>  -   <u>EXPRESSION OF THE HIV-1 (ARV-2 OR SF-2
                       STRAIN) env GENE IN ALVAC, TROVAC AND
                       NYVAC VECTORS</u>

<u>Plasmid Constructions</u>

5           The lambda clone containing the entire HIV-1
      (ARV-2 or SF-2 strain) genome was provided by J. Levy and
      was described previously (Sanchez-Pescador et al., 1985).
      The <u>env</u> sequences were subcloned into pUC13, creating
      plasmid pMP7MX373, which contains the sequences from -1

10    relative to the initiation codon (ATG) of the <u>env</u> gene
      product to 715 bp downstream of the termination codon
      (TAA) of the <u>env</u> gene.   These <u>env</u> sequences were excised
      from pMP7MX373 by digestion with <u>EcoRI</u> and <u>HindIII</u> and
      inserted into the plasmid vector, pIBI25 (International

15    Biotechnologies, Inc., New Haven, CT) generating plasmid
      pIBI25env.

            Recombinant plasmid pIBI25<u>env</u> was used to
      transform competent <i>E. coli</i> CJ236 (dut- ung-) cells.
      Single-stranded DNA was isolated from phage derived by

20    infection of the transformed <i>E. coli</i> CJ236 cells with the
      helper phage, MG408.  This single-stranded template was
      used in <i>in vitro</i> mutagenesis reactions (Kunkel et al.,
      1985) with oligonucleotide MUENVT12 (SEQ ID NO:45) (5'-
      AGAGGGGAATTCTTCTACTGCAATACA-3').  Mutagenesis with this

25    oligonucleotide generates a T to C transition and
      disrupts the T5CT motif at nucleotide positions 6929-6935
      of the ARV-2 genome (Sanchez-Pescador et al., 1985).
      This mutation does not alter the amino acid sequence of
      the <u>env</u> gene and creates an <u>EcoRI</u> site, which was used to

30    screen for mutagenized plasmid clones.  Sequence
      confirmation was done by the dideoxynucleotide chain
      termination method (Sanger et al., 1977).  The resultant
      mutagenized plasmid was designated as pIBI25mut<u>env</u>11.

            A 1.45 kb <u>BglII</u> fragment was derived from

35    pIBI25mut<u>env</u>11.  This fragment contained the mutated <u>env</u>
      sequences.  It was used to substitute for the
      corresponding unmutated fragment in pIBI25<u>env</u>.  The

resultant plasmid was designated as pIBI25mut_env_8.
Further modifications were made to pIBI25mut_env_8.  *In
vitro* mutagenesis was performed to remove the sequence
coding for the rex protein and the LTR sequence (LTR
5   region) from the 3'-end of the gene and to delete the
putative immuno-suppressive (IS) region amino acids 583
through 599 (SEQ ID NO:46:Leu-Gln-Ala-Arg-Val-Leu-Ala-
Val-Glu-Arg-Tyr-Leu-Arg-Asp-Gln-Gln-Leu) (Klasse et al.,
1988).  These reactions were done with the single-
10  stranded template derived from pIBI25mut_env_8 with
oligonucleotides LTR2 (SEQ ID NO:47) (5'-
TTGGAAAGGCTTTTGGCATGCCACGCGTC-3') and MUENSVISR (SEQ ID
NO:48) (5'-ACAGTCTGGGGCATCAAGCAGCTAGGGATTTGGGGTTGCTCT-
3').  Mutagenized clones were identified by hybridization
15  and restriction analysis.  A clone mutagenized such that
it was deleted both of the IS and the LTR region and
another deleted of the LTR was confirmed by nucleotide
sequence analysis and designated pIBI25mut3_env_40 and
pIBI25mut2_env_22, respectively.
20          A 3.4 kb _SmaI_/_HindIII_ fragment containing the
entire _env_ gene was derived from pIBI25mut3_env_40 and from
pIBI25mut2_env_22 and inserted into pCPCV1 and pFPCV2,
digested with _SmaI_/_HindIII_.  The plasmid pCPCV1 is an
insertion plasmid which enables the generation of ALVAC
25  recombinants with insertion occurring in the C3 locus.
The plasmid, pFPCV2, is an insertion plasmid which
enables the generation of TROVAC recombiants with
insertion occurring in the F7 locus.  Plasmids pCPCV1 and
pFPCV2 have been described previously in PCT
30  International Publication No. WO 89/03429 published April
20, 1989.
            Oligonucleotide PROVECNS (SEQ ID NO:49) (5'-
CCGTTAAGTTTGTATCGTAATGAAAGTGAAGGGGACCAGG-3') was used for
*in vitro* mutagenesis reactions via the method of Mandecki
35  (1986) to make a precise ATG:ATG construction with the
VVH6 promoter and the _env_ sequences.  Potential mutants
were screened for the loss of the _SmaI_ site.  Plasmid

clones devoid of a SmaI site were identified and
confirmed by nucleotide sequence analysis. Properly
mutagenized plasmid clones were identified and designated
as pCPenvIS+ or pCPenvIS- and pFPenvIS+ or pFPenvIS.

5          The HIV-1 env genes were excised from pCPenvIS-
by digestion with NruI and HindIII. The two env
fragments of 2.5 kb (envIS+) and 2.4 kb (envIS-),
respectively, were isolated and blunt-ended by reaction
with the Klenow fragment of the E. coli DNA polymerase in
10  the presence of 2 mM dNTPs. These fragments were ligated
with the 3.5 kb fragment derived by digestion of
pSIVenvVV with NruI and PstI with a subsequent blunting
step with the Klenow fragment of the E. coli DNA
polymerase in the presence of 2mM dNTPs. The plasmid
15  pSIVenvVV contains the SIV env gene expression cassette
regulated by the vaccinia virus H6 promoter in the ATI
insertion locus. Digestion of pSIVenvVV with NruI and
PstI excises the entire SIV env coding sequences and the
3'-most 20 bp of the promoter element. Ligation to the
20  env IS- and env IS+ fragments restores the 20 bp of the
H6 promoter and inserts the HIV-1 env gene into the ATI
insertion plasmid. The resultant plasmids were
designated as pAR5VV+ and pAR6VV- for env IS+ and env IS,
respectively.

25  *In Vitro Recombination and Purification of Recombinants*
           Recombination was performed introducing plasmid
DNA into infected cells by calcium phosphate
precipitation both for ALVAC and for TROVAC recombinants,
as previously described (Piccini et al., 1987). Plasmids
30  pCPenvIS+ and pCPenvIS- were used to make recombinants
vCP61 and vCP60, respectively. Plasmids pFPenvIS+ and
pFPenvIS- for recombinants vFP63 and vFP62, respectively.
The plasmids pAR5VV+ and pAR6VV- were used in *in vitro*
recombination experiments with vP866 as rescue to yield
35  vP939 and vP940, respectively. Recombinant plaques were
selected by autoradiography after hybridization with a
$^{32}$P-labeled env specific probe and passaged serially three

51

times to assure purity, as previously described (Piccini
et al., 1987).

**Expression of the HIV-1 env Gene**

Six different recombinant viruses were prepared
5   where the HIV env gene of the ARV-2 or SF-2 strain was
inserted downstream from a vaccinia early-late promoter,
H6.  For simplicity, the two ALVAC-based recombinant
viruses, vCP61 and vCP60, will be referred to as CPIS+
and CPIS-, the two TROVAC-based recombinants, vFP63 and
10  vFP62, as FPIS+ and FPIS-, and the two NYVAC-based
recombinants, vP939 and vP940, as VV- and VV+,
respectively.

All the constructs were precise, in that, the
ATG initiation codon of the HIV-1 env gene was
15  superimposed on the ATG of the vaccinia H6 promoter.
Moreover, all extraneous genetic information 3' to the
termination codon was eliminated.  CPIS-, FPIS-, and VV-
were all obtained by deletion of a 51 bp region,
corresponding to amino acids 583-599, located near the 5'
20  portion of the gp41 gene product.  This region shares
homology with putative immunosuppressive regions (Klasse
et al., 1988; Ruegg et al., 1989a,b) occurring in the
transmembrane polypeptide of other retrovirus
glycoproteins (Cianciolo et al., 1985; Ruegg et al.,
25  1989a,b).

Expression analyses with all six recombinant
viruses were performed in CEF, Vero, and MRC-5 cell
monolayers.  Radioimmunoprecipitation experiments using
pooled sera from HIV seropositive individuals were
30  performed as described above.  All six recombinants
directed the synthesis of the HIV-1 gp160 envelope
precursor.  The efficiency of processing of gp160 to
gp120 and gp41, however, varied between cell types and
was also affected by deletion of the immunosuppressive
35  region.  Recognition of gp41 by the pooled sera from HIV
seropositive individuals also varied between the virus
background and the cell type.

52

**Example 4  -  EXPRESSION OF THE HIV-2 (ISSY STRAIN) env GENE IN NYVAC**

**Expression of gp160**

Oligonucleotides HIV25PA (SEQ ID NO:50) (5'-

5  ATGAGTGGTAAAATTCAGCTGCTTGTTGCCTTTCTGCTAACTAGTGCTTGCTTA-

3') and HIV25PB (SEQ ID NO:51) (5'-

TAAGCAAGCACTAGTTAGCAGAAAGGCAACAAGCAGCTGAATTTTACCACTCAT-

3') were annealed to constitute the initial 54 bp of the

HIV-2 ISSY strain (Franchini et al., 1989) env coding

10  sequence.  This fragment was fused 3' to a 129 bp

fragment derived by PCR with oligonucleotides H65PH (SEQ

ID NO:52) (5'-ATCATCAAGCTTGATTCTTTATTCTATAC-3') and

H63PHIV2 (SEQ ID NO:53)

(5'-CAGCTGAATTTTACCACTCATTACGATACAAACTTAACG-3') using

15  pTP15 (Guo et al., 1989) as template.  The fusion of

these two fragments was done by PCR using

oligonucleotides HIV25PC (SEQ ID NO:54) (5'-

TAAGCAAGCACTAGTTAG-3') and H65PH (SEQ ID NO:52).  The 174

bp PCR derived fragment was digested with HindIII and

20  SacI and inserted into pBS-Sk (Stratagene, La Jolla, CA)

digested with HindIII and SacI.  The resultant fragment

was designated pBSH6HIV2.  The insert was confirmed by

nucleotide sequence analysis.

The 3' portion of the HIV-2 env gene was also

25  derived by PCR.  In this reaction a 270 bp fragment was

amplified with oligonucleotides HIV2B1 (SEQ ID NO:55)

(5'-CCGCCTCTTGACCAGAC-3') and HIV2B2 (SEQ ID NO:56) (5'-

ATCATCTCTAGAATAAAAATTACAGGAGGGCAATTTCTG-3') using pISSY-

KPN (provided by Dr. Genoveffa Franchini, NCI-NIH,

30  Bethesda, MD) as template.  This fragment fragment was

digested with BamHI and XbaI.  The 150 bp fragment

derived from this digestion contained a 5' BamHI and a 3'

XbaI cohesive end.  The fragment was engineered to

contain a T5NT sequence motif known to be recognized as

35  vaccinia virus early transcription termination signal

(Yuen et al., 1987), following the termination codon

(TAA).

The majority of the HIV-2 _env_ gene was obtained
from pISSY-KPN by digestion with SacI and BamI.  The 2.7
kb fragment generated by this digestion was coinserted
into pBS-SK digested with SacI and XbaI with the 150 bp
5   BamHI/XbaI fragment  corresponding to the 3' end of the
gene.  The resultant plasmid  was designated pBSHIV2ENV.
      The 174 bp SpeI/HindIII fragment from pBSH6HIV2
and the 2.5 kb SpeI/XbaI fragment from pBSHIV2ENV were
ligated into pBS-SK digested with HindIII and XbaI to
10  yield pBSH6HIV2ENV.  The 2.7 kb HindIII/XbaI insert from
pBSH6HIV2ENV was isolated and blunt-ended with the Klenow
fragment of the _E. coli_ DNA polymerase in the presence of
2mM dNTP.  The blunt-ended fragment was inserted into a
SmaI digested pSD541VC insertion vector.  The resultant
15  plasmid was designated as pATIHIV2ENV.  This plasmid was
used _in vitro_ recombination experiments with vP866
(NYVAC) as the rescuing virus to yield vP920.
      Immunoprecipitation analysis was performed to
determine whether vP920 expresses authentic HIV-2 gp160.
20        Lysates from the infected cells were analyzed
for HIV-2 _env_ gene expression using pooled serum from
HIV-2 seropositive individuals (obtained from Dr.
Genoveffa Franchini, NCI-NIH, Bethesda, MD).  The sera
was preadsorbed with vP866 infected Vero cells.  The
25  preadsorbed human sera was bound to Protein A-sepharose
in an overnight incubation at 4°C.  Following this
incubation period, the material was washed 4X with 1X
buffer A.  Lysates precleared with normal human sera and
protein A-sepharose were then incubated overnight at 4°C
30  with the human sera from seropositive individuals bound
to protein A-sepharose.  After the overnight incubation
period, the samples were washed 4X with 1X buffer A and
2X with a LiCl$_2$/urea buffer.  Precipitated proteins were
dissociated from the immune complexes by the addition of
35  2X Laemmli's buffer (125mM Tris(pH6.8), 4% SDS, 20%
glycerol, 10% 2-mercaptoethanol) and boiling for 5 min.
Proteins were fractionated on a 10% Dreyfuss gel system

(Dreyfuss et al., 1984), fixed and treated with 1M Na-
salicylate for fluorography.

Human sera from HIV-2 seropositive individuals
specifically precipitated the HIV-2 gp160 envelope

5  glycoprotein from vP920 infected cells. Furthermore, the
authenticity of the expressed HIV-2 env gene product was
confirmed, since the gp160 polyprotein is processed to
the mature gp120 and gp41 protein species. No HIV-
specific protein species were precipitated from mock-

10  infected cells or cells infected with the parental virus,
vP866. Also, supportive of the proper expression of the
HIV-2 env by vP920 was the observation by an
immunofluorescence assay that the gene product is
expressed on the surface of vP920 infected cells.

15  **Expression of gp120**

The plasmid pBSH6HIV2 containing the vaccinia
virus H6 promoter fused to the 5'-end of the HIV-2 env
gene was digested with SpeI and HindIII to liberate the
180 bp fragment containing these sequences. This

20  fragment was ligated into pBS-SK digested with HindIII
and XbaI along with the 1.4 kb SpeI/XbaI fragment of
pBSHIV2120A to yield pBSHIV2120B.

The plasmid pBSHIV2120A was derived by
initially deriving the 3' portion of the gp120 coding

25  sequence by PCR. The PCR was performed using
oligonucleotides HIV2120A (SEQ ID NO:57) (5'-
ATCATCTCTAGAATAAAAATTATCTCTTATGTCTCCCTGG-3') and HIV2120B
(SEQ ID NO:58) (5'-AATTAACTTTACAGCACC-3') with pISSY-KPN
as template. The PCR-derived fragment was digested with

30  EcoRI and XbaI to yield a 300 bp fragment which contained
a 5'-EcoRI cohesive end and a 3'-XbaI cohesive end. The
fragment was engineered with a translation termination
sequence (TAA) and a T5NT sequence motif just 5' to the
XbaI site. The 300 bp XbaI/EcoRI PCR fragment was

35  ligated into pBS-SK digested with SacI/XbaI along with a
1.4 kb SacI/EcoRI fragment derived from pISSY-KPN to
generate pBSHIV2120A.

The plasmid pBSHIV2120B was digested with
HindIII and XbaI to generate a 1.8 kb fragment containing
the HIV-2 gp120 coding sequence juxtaposed 3' to the
vaccinia virus H6 promoter.  This fragment was blunted
5   with the Klenow fragment of the *E. coli* DNA polymerase in
the presence of 2 mM dNTPs.  The blunt-ended fragment was
ligated to SmaI digested pSD541VC to generate
pATIHIV2120.  This plasmid was used in *in vitro*
recombination experiments to yield vP922.

10          Immunoprecipitation experiments with vP922
infected cells were performed as described above for the
expression of the entire HIV-2 env gene.  No HIV-specific
species were precipitated from mock infected or vP866
infected Vero cells.  A protein species of 120 kDa was,
15  however, precipitated from lysates derived from cells
infected with vP922.

**Example 5  -  EXPRESSION OF SIV GENES IN NYVAC**
**Generation of NYVAC/SIV gp140 Recombinant**

            A plasmid pSS11E containing the SIV (mac142) env
20  gene was obtained from Dr. Genoveffa Franchini (NCI-NIH,
Bethesda, MD).  This plasmid was digested with HindIII
and PstI to liberate a 2.2 kbp fragment containing from
nucleotide 220 of the SIV env gene to a region 160 bp
downstream from the translation termination codon.  It
25  should be noted that an expression cassette containing
this fragment will result in the expression of a gp140
protein species rather that a gp160 species.  This 40%
deletion of the transmembrane region results from a
premature termination at nucleotide 7,934 of the genome
30  (Franchini et al., 1987).  Such premature terminations of
the env gene product are noted after propagation of SIV
in culture (Kodama et al., 1989).

            The amino portion of the gene was derived by
PCR using pSS11E as template and oligonucleotides SIVENV1
35  (SEQ ID NO:59) (5'-
CGATATCCGTTAAGTTTGTATCGTAATGGGATGTCTTGGGAATC-3') and
SIVENV2 (SEQ ID NO:60) (5'-CAAGGCTTTATTGAGGTCTC-3').  The

resultant 250 bp fragment contains the 5'-most 230 bp of
the SIV env gene juxtaposed downstream from the 3'-most
20 bp of the vaccinia virus H6 promoter (3'-end of NruI
site). A 170 bp fragment was obtained by digestion of
5    the fragment with HindIII, which removes 80 bp of SIV env
sequences.

     The sequences containing the remainder of the
SIV env gene following the premature termination signal
were derived by PCR from pSS35E (obtained from Dr.
10   Genoveffa Franchini). This plasmid contains sequences
containing the C-terminal portion of the SIV env gene
into the LTR region downstream from the env gene. The
oligonucleotides used to derive the 360 bp fragment were
SIVENV3 (SEQ ID NO:61) (5'-CCTGGCCTTGGCAGATAG-3') and
15   SIVENV4A (SEQ ID NO:62) (5'-
ATCATCGAATTCAAAAATATTACAAAGAGCGTGAGCTCAAGTCCTTGCCTAATCCTC
C-3'). This fragment was digested with PstI and EcoRI to
generate a 260 bp fragment having a 5' PstI cohesive end
and a 3'- EcoRI cohesive end.
20       The 2.2 kb HindIII/PstI fragment from pSS11E,
the 170 bp NruI/HindIII fragment containing the 5' end of
the gene, and the 260 bp PstI/EcoRI containing the 3' end
of the gene were ligated with a 3.1 kb NruI/EcoRI
fragment derived from pRW838. pRW838 contains the
25   vaccinia virus H6 promoter linked to the rabies G gene
flanked by canarypoxvirus sequences which enable the
insertion of genes into the C5 locus. Digestion with
NruI and EcoRI liberates the rabies G gene and removes
the 3'-most 20 bp of the H6 promoter. The resultant C5
30   insertion plasmid containing the SIV env gene linked to
the vaccinia H6 promoter was designated as pC5SIVENV.
     The plasmid, pC5SIVENV, was digested with
HindIII and EcoRI to liberate a 2.2 kb fragment,
containing from nucleotide 150 of the SIV env gene to the
35   end of the entire gene. PCR was used to derive the
vaccinia H6 promoter/SIV env linkage from pC5SIVENV with
oligonucleotides MPSYN286 (SEQ ID NO:63) (5'-

CCCCCCAAGCTTTTTTATTCTATACTT-3') and SIVENV2 (SEQ ID
NO:64) (5'-CAAGGCTTTATTGAGGTCTC-3'). The 320 bp fragment
was digested with HindIII to derive a 240 bp fragment.
The 2.2 kb HindIII/EcoRI and the 240 bp HindIII fragment

5    were coligated into pC3I digested with HindIII and EcoRI.
The resultant plasmid containing the HindIII fragment in
the proper orientation relative to the SIV env coding
sequence was designated pC3SIVEM. The plasmid pC3I was
derived as follows. The nucleotide sequence analysis of

10   an 2.5 kb BglII canarypoxvirus genomic fragment revealed
the entire C3 open reading frame and the 5' and 3'
flanking regions. In order to construct a donor plasmid
for insertion of foreign genes into the C3 locus with the
complete excision of the C3 open reading frame, PCR

15   primers were used to amplify the 5' and 3' sequences
relative to C3. Primers for the 5' sequences were RG277
(SEQ ID NO:65) (5'-CAGTTGGTACCACTGGTATTTTATTTCAG-3') and
RG278 (SEQ ID NO:66) (5'-
TATCTGAATTCCTGCAGCCCGGGTTTTTATAGCTAATTAGTCAAATGTGAGTTAATA

20   TTAG-3').
        Primers for the 3' sequences were RG279 (SEQ ID
NO:67) (5'-
TCGCTGAATTCGATATCAAGCTTATCGATTTTTATGACTAGTTAATCAAATAAAAAG
CATA CAAGC-3') and RG280 (SEQ ID NO:68) (5'-

25   TTATCGAGCTCTGTAACATCAGTATCTAAC-3'). The primers were
designed to include a multiple cloning site flanked by
vaccinia transcriptional and translational termination
signals. Also included at the 5'-end and 3'-end of the
left arm and right arm were appropriate restriction sites

30   (Asp718 and EcoRI for left arm and EcoRI and SacI for
right arm) which enabled the two arms to ligate into
Asp718/SacI digested pBS-SK plasmid vector. The
resultant plasmid was designated as pC3I.
        The plasmid pC3SIVEM was linearized by

35   digestion with EcoRI. Subsequent partial digestion with
HindIII liberated a 2.7 kb HindIII/EcoRI fragment. This
fragment was blunt-ended by treatment with Klenow

58

fragment of the *E. coli* DNA polymerase in the presence of 2mM dNTPs. The fragment was ligated into pSD550VC digested with *SmaI*. The resultant plasmid was designated as pSIVEMVC. This plasmid was used in *in vitro*

5  recombination experiments with vP866 as rescue virus to generate vP873. vP873 contains the SIV env gene in the I4L locus.

**Generation of a NYVAC/gag/pol and gag Recombinants**

A plasmid, pSIVAGSS11G, containing the SIV cDNA

10  sequence encompassing the gag and pol genes was obtained from Dr. Genoveffa Franchini (NCI-NIH, Bethesda, MD). The gag and pol genes from this plasmid were juxtaposed 3' to the vaccinia I3L promoter between vaccinia tk flanking arms. This was accomplished by cloning the

15  4,800 bp CfoI/TaqI fragment of pSIVGAGSS11G, containing the gag and the oligonucleotides SIVL1 (SEQ ID NO:69) (5'-TCGAGTGAGATAAAGTGAAAATATATATCATTATATTACAAGTA CAATTATTTAGGTTTAATCATGGGCG-3') and SIVL2 (SEQ ID NO:70) (5'-

20  CCCATGATTAAACCTAAATAATTGTACTTTGTAATATAATGCTATATATTTTCACTT TATCTCAC-3') corresponding to the I3L promoter into the 4,070 bp XhoI/AccI fragment of pSD542, a derivative of pSD460 (Fig. 1). The plasmid generated by this manipulation was designated pSIVG1.

25  To eliminate the pol gene, a 215 bp PCR fragment was derived from pSIVGAGSS11G using oligonucleotides SIVP5 (SEQ ID NO:71) (5'-AATCAGAGAGCAGGCT-3') and SIVP6 (SEQ ID NO:72) (5'-TTGGATCCCTATGCCACCTCTCT-3'). The PCR-derived fragment

30  was digested with *BamHI* and *StuI* and ligated with the 5,370 bp partial *BamHI/StuI* fragment of SIVG1. This resulted in the generation of pSIVG2. pSIVG2 was used in *in vitro* recombination experiments with vP873 as rescue virus to yield vP948.

35  The plasmid to insert both gag and pol into NYVAC-based vectors was engineered in the following manner. pSIVG1, described above, contains extraneous 3'-

59

noncoding sequences which were eliminated using a 1 kb
PCR fragment.  This fragment was generated from plasmid
pSIVGAGSS11G with the oligonucleotides SIVP5 and SIVP6.
This PCR derived fragment containing the 3' end of the

5  pol gene was digested with BamHI and HpaI.  The 1 kb
BamHI/HpaI fragment was ligated to the 7,400 bp partial
BamHI/HpaI fragment of pSIVG1 to yield pSIVG4.

       Sequence analysis of pSIVG4 revealed a single
base pair deletion within the pol gene.  To correct this

10  error the 2,300 bp BglII/StuI fragment from pSIVG1 was
inserted into the 6,100 bp partial BglII/StuI fragment of
pSIVG4 to yield pSIVG5.  The plasmid, pSIVG5, was used in
in vitro recombination experiments with vP873 as rescue
to generate vP943.

15  **Generation of NYVAC/SIV p16 and p28 Recombinants**

       The pol gene and the portion of the gag gene
encoding p28, p2, p8, p1, and p6 were eliminated from
pSIVG1.  This was accomplished by cloning the
oligonucleotides SIVL10 (SEQ ID NO:73) (5'-

20  AGACCAACAGCACCATCTAGCGGCAGAGGAGGAAATTACTAATTTTTATTCTAGAG-
3') and SIVL11 (SEQ ID NO:74) (5'-
GATCCTCTAGAATAAAAATTAGTAATTTCCTC
CTCTGCCGCTAGATGGTGCTGTTGGT-3') into the 4,430 bp
AccI/BamHI fragment of pSIVG1 to generate pSIVG3.  This

25  plasmid contains an expression cassette for the SIV p16
gene product expressed by the vaccinia I3L promoter.

       The entomopoxvirus 42 kDa-promoted SIV p28 gene
(5' end only) was inserted downstream from the I3L-
promoted p16 gene.  This was accomplished by cloning the

30  360 bp BspMI/BamHI fragment of pSIVG1, containing the 5'
end of the p28 gene, the oligonucleotides pSIVL14 (SEQ ID
NO:75) (5'-
TAGACAAAATTGAAAATATATAATTACAATATAAAATGCCAGTACAACAAATAGGTG
GTAACTATGTCCACCTGCCATT-3') and SIVL15 (SEQ ID NO:76) (5'-

35  GCTTAATGGCAGGTGGACATAGTTACCACCTATTTGTTGTACTGGCATTTTATATTG
TAATTATATATTTTCAATTTTGT-3'), containing the entomopox 42
kDa promoter into the 4,470 bp partial XbaI/BamHI

fragment of pSIVG3. The resultant plasmid was designated
as pSIVG6.

The 3' portion of the p28 gene was then
inserted into pSIVG6. A 290 bp PCR fragment, containing
5   the 3' end of the SIV p28 gene, was derived from pSIVGI
using oligonucleotides SIVP12 (SEQ ID NO:77) (5'-
TGGATGTACAGACAAC-3') and SIVP13 (SEQ ID NO:78) (5'-
AAGGATCCGAATTCTTACATTAATCTAGCCTTC-3'). This fragment was
digested with BamHI and ligated to the 4,830 bp BamHI
10  fragment of pSIVG6. The resultant plasmid, pSIVG7, was
used in in vitro recombination experiments with vP866 and
vP873 as rescue experiments to generate vP942 and vP952,
respectively.

Expression Analyses

15      The SIV gp140 env gene product is a typical
glycoprotein associated with the plasma membrane of
infected cells. It is expressed as a polyprotein of 140
kDa that is proteolytically cleaved to an extracellular
species of 112 kDa and a transmembrane region of 28 kDa
20  (Franchini et al., 1987). Immunofluorescence analysis
using sera from rhesus macaques seropositive for SIV
followed by fluorescein conjugated rabbit anti-monkey IgG
demonstrated expression of the env gene product on the
surface of recombinant infected Vero cells. Surface
25  expression was not detectable on the surface of mock
infected cells or cells infected with the NYVAC (vP866)
parent virus. Furthermore, cells infected with
recombinants containing only gag genes were not shown to
express any SIV components on the surface. Surface
30  expression in cells infected with vP873, vP943, vP948 and
vP952 all demonstrated surface expression and
significantly, all contain the SIV env gene.

The authenticity of the expressed SIV gene
products (env and gag) in Vero cells infected with the
35  NYVAC/HIV recombinants was analyzed by
immunoprecipitation as described above, except all
samples were harvested at 17 hours post infection by the

addition of 1 ml of 3X Buffer A. Lysates from the
infected cells were analyzed with pooled sera from SIV
seropositive rhesus macaques or a monoclonal antibody
specific for gag p24 gene product (both obtained from Dr.
5   Genoveffa Franchini, NCI-NIH, Bethesda MD).

Immunoprecipitation with the SIV seropositive
macaques sera was performed in the following manner. The
macaque sera was incubated with a protein A-sepharose at
4°C for 16 hours. After washing with buffer A, the sera
10  bound to protein A sepharose was added to lysates
precleared with normal monkey sera and protein A
sepharose. Following an overnight incubation at 4°C the
precipitates were washed 4x with buffer A and 2 x with
LiCl/urea buffer. To dissociate the precipitated protein
15  from the antibody, the samples were boiled in 80 $\mu$l 2 x
Laemmli buffer for 5 minutes. The samples were
fractionated on a 12.5% gel using the Dreyfuss gel system
(Dreyfuss et al., 1984). The gel was fixed and treated
with 1M Na-salicylate for fluorography. All the
20  recombinants containing SIV genes were expressing the
pertinent gene products. The NYVAC recombinants vP873,
vP943, vP948 and vP952 which contain the SIV env gene all
expressed the authentic gp140. However, it is difficult
to assess the processing of the gp140 protein to the 112
25  kDa and 28 kDa mature forms. No species with an apparent
molecular weight of 140 kDa was precipitated by macaque
anti-SIV sera from mock infected Vero cells, vP866
infected Vero cells and Vero cells infected with
NYVAC/SIV recombinants not containing the SIV env gene.
30  Expression of the SIV gag encoded gene products by vP942,
vP943, vP948, and vP952 was demonstrated using the pooled
sera from macaques infected with SIV and the monoclonal
antibody specific to the p28 gag component. Expression
of the entire p55 gag protein without the pol region,
35  which contains the protease function, by NYVAC (vP948) in
Vero cells is evident. These results demonstrate that
lack of SIV protease expression prevents the complete

proteolysis of p55 into its mature form. This is demonstrated much more clearly when a monoclonal antibody specific to p28 was used to precipitate gag specific gene products from vP948 infected Vero cells. Contrary to
5 this result, expression of SIV gag with the pol gene (includes protease in vP943 infected Vero cells) enabled the expressed p55 gag precursor polypeptide to be proteolytically cleaved to its mature forms.

Expression of both the p16 and p28 SIV gene
10 products in vP942 and vP952 infected Vero cells was demonstrated using the pooled sera from macaques infected with SIV. Using the monoclonal antibody specific to p24 obviously only recognized the p28 expressed component.

Certain Materials and Methods Which Relate to
15 Examples 6 to 8.

Assay for Cytotoxic T Lymphocytes and In Vitro Stimulation of Memory Precursors of Cytotoxic T Lymphocytes. Six week old female BALB/c mice were inoculated intravenously with 5x10$^7$ pfu of vaccinia virus
20 (NYVAC), recombinant vaccinia virus expressing HIV-1 (IIIB) env (vP911), canarypoxvirus (ALVAC), or with recombinant canarypoxvirus expressing HIV-1 (IIIB) env (vCP112). Seven days later, the spleen cells were assayed for primary CTL activity against unmodified P815
25 cells or P815 cells that had been incubated overnight with a peptide corresponding to the hypervariable V3 loop region of HIV-1 (IIIB) gp120. Twenty-two days after the initial immunization, the spleen cells of the experimental mice were incubated with poxvirus infected
30 stimulator spleen cells and assayed for memory CTL activity against peptide pulsed targets as before. To determine secondary CTL activity, 29 days after the primary immunization mice received a second inoculation of identical dosage and content as the first. Five days
35 later, the spleen cells were assayed for cytolytic activity against peptide pulsed targets. For cytotoxicity assays, H-2$^d$ P815 murine mastocytoma cells

were incubated overnight in medium (Minimum Essential
Medium containing Earle's salts and supplemented with 10%
fetal bovine serum, 2 mM L-glutamine, 100 U/ml
penicillin, and 100 μg/ml streptomycin) with or without

5    20 μg/ml V3 peptide (CNTRKRIRIQRGPGRAFVTGK, American Bio-
     Technologies, Inc.) (SEQ ID NO:79). The following
     morning, the P815 cells were washed by centrifugation and
     labeled for 1 hr at 37°C in 100 μCi of $Na_2{}^{51}CrO_4$ per $2x10^6$
     cells. Intact spleens were aseptically removed from

10   euthanized mice, bathed in ice cold Hank's Balanced Salt
     Solution, and disrupted into single cell suspensions
     using a Stomacher blender. The spleen cell suspensions
     were washed several times by low speed centrifugation and
     resuspended in Assay Medium (RPMI 1640 containing 10%

15   fetal bovine serum, 20 mM HEPES, 2 mM L-glutamine, $5x10^{-5}$
     M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml
     streptomycin). For memory CTL activity, the spleen cells
     from immunized mice were resuspended in Stimulation
     Medium (Minimum Essential Medium with Earle's salts

20   containing 10% fetal bovine serum, 2mM L-glutamine, $10^{-4}$M
     2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml
     streptomycin) and stimulated in vitro in upright 25 $cm^2$
     tissue culture flasks with naive syngeneic stimulator
     spleen cells that had been infected with one of the

25   poxviruses or poxvirus recombinants. After five days at
     37°C, the cells were washed, counted, and resuspended in
     Assay Medium. $^{51}$Chromium labelled target cells were added
     to titrated effector cells in 96-well microtiter plates
     for a 4 hr $^{51}$Cr release assay. Effector to target cell

30   ratios (E:T) shown for the three assays were 100:1
     (primary), 20:1 (memory), and 50:1 (secondary). Percent
     cytotoxicity was calculated as described above, i.e.,
     (experimental $^{51}$Cr release - spontaneous $^{51}$Cr
     release)/(maximum $^{51}$Cr release - spontaneous $^{51}$Cr release)

35   x 100. Maximum release was determined by the addition of
     5% sodium dodecyl sulfate to target cells while
     spontaneous release was determined by incubating target

64

cells in the absence of effector cells. In none of the
experiments presented did spontaneous release of $^{51}$Cr from
target cells exceed 20% of maximum $^{51}$Cr release. Error
bars in Fig. 7 represent 1 standard deviation from the
5  mean. $P<0.05$, Student's $t$-test compared to appropriate
vaccinia or canarypoxvirus immunized mice.

        Cell Surface Phenotype of Cytotoxic Effector
Cells. Mice spleen cells were immunized with vaccinia
virus or canarypox virus vectors (NYVAC, ALVAC) or with
10  vaccinia virus or canarypox virus recombinants expressing
HIV IIIB env (vP911, vCP112). A second inoculation was
administered 30 days after the first. Prior to addition
to V3 peptide pulsed targets, the spleen cells were
treated with monoclonal antibodies or alloantiserum to
15  murine T-lymphocyte surface antigens in a two-stage
protocol. Briefly, the spleen cells were resuspended at
$10^7$ viable cells per ml of Cytotoxicity Medium (RPMI 1640
containing 0.2% BSA and 5 mM HEPES) to which was added
alloanti-Thy 1.2 (Cedarlane), monoclonal anti-CD4 (172.4,
20  K.J. Weinhold, Duke University Medical Center), or
monoclonal anti-Lyt 2.2 (Cedarlane). After 30 min at
5°C, the cells were washed and resuspended in the
original volume of Cytotoxicity Medium, divided into two
equal portions with or without complement (Rabbit Lo-Tox
25  M, Cedarlane) and incubated at 37°C for 45 min. The
cells were then washed in Assay Medium and, based on the
pre-treatment cell densities, resuspended in volumes of
Assay Medium approximating effector to target cell ratios
of 100:1 (primary), 10:1 (memory), or 80:1 (secondary)
30  before addition to a 5 hr $^{51}$Cr release assay. Error bars
in Fig. 10 represent 1 standard deviation from the means.

        Specificity of CTL Antigen Receptor Recognition
of the V3 Loop Region of HIV IIIB gp120. Cytotoxic T
lymphocytes and memory precursors of cytotoxic T
35  lymphocytes were generated by inoculation of mice with
vCP112 as described above. Assays for cytotoxic T
lymphocytes were performed as described above except that

P815 target cells were pulsed overnight with V3 peptide
from HIV-1 IIIB (CNTRKRIRIQRGPGRAFVTGK) (SEQ ID NO:79),
MN (CNKRKRIHIGPGRAFYTTKN) (SEQ ID NO:80), or SF2
(CNTRKSIYIGPGRAFHTTGR) (SEQ ID NO:81). Effector to
5   target cell ratios were 100:1 (primary), 20:1 (memory),
and 50:1 (secondary).

      Antibody Responses to HIV-1 (IIIB) gp120. The
wells of ELISA plates (Immulon II) were coated overnight
at 4°C with 0.5 $\mu$g of partially purified HIV-1 (IIIB)
10  gp120 (Dr. G. Franchini, NCI-NIH, Bethesda, MD) in
carbonate buffer, pH 9.6. The plates were then washed
with phosphate-buffered saline containing 0.05% Tween 20
(PBST). The plates were then blocked for 2 hr at 37°C
with PBST containing 1% bovine serum albumin (BSA).
15  After washing with PBST, sera were initially diluted 1:20
with PBST containing 0.1% BSA (dilution buffer). The
sera were further 2-fold serially diluted in the wells of
the ELISA plate. The plates were incubated at 37°C for 2
hr and washed with PBST. Horseradish peroxidase
20  conjugated rabbit anti-mouse immunoglobulins (DAKO) was
diluted 1:2000 in dilution buffer and added to the wells
of the ELISA plated and incubated at 37°C for 1 hour.
After washing with PBST, OPD (o-phenylenediamine
dihydrochloride) in substrate buffer was added and the
25  color was allowed to develop at ambient temperature for
about 20 min. The reaction was extinguished by the
addition of 2.5 M $H_2SO_4$. The absorbance at 490 nm was
determined on a Bio-Tek EL-309 ELISA reader. The serum
endpoint was defined as the reciprocal of the dilution
30  giving an absorbance value of 0.4.

    **Example 6   -   A RECOMBINANT CANARYPOXVIRUS EXPRESSING**
                        **HIV env ELICITS HIV-SPECIFIC CYTOTOXIC T**
                        **LYMPHOCYTE ACTIVITY**

      Seven days after the initial inoculation with
35  the HIV canarypoxvirus recombinant (vCP112; defined in
Example 2), cytotoxic responses of spleen cells against
HIV V3 peptide pulsed target cells were roughly

66

equivalent to the cytotoxic responses elicited by the
same dose, 5x10⁷ pfu, of the NYVAC recombinant, vP911
(Example 2) expressing the same HIV _env_ gene (Fig. 7).
Following appropriate _in vitro_ stimulation or a second

5    inoculation, the levels of cytotoxicity of the spleen
cells of mice given the canarypoxvirus recombinant
increased and were comparable to spleen cells from mice
similarly administered the NYVAC recombinant.  No such
cytotoxic responses were detected from spleen cells of

10   mice inoculated with the non-recombinant NYVAC or ALVAC
vectors confirming the requirement for immunization with
a poxvirus recombinant expressing the HIV _env_ gene.
Furthermore, no cytotoxic reactivity was detected against
unmodified P815 cells from the spleen cells of any of the

15   mice regardless of the inoculation regimen.  Thus, only
mice inoculated with recombinant NYVAC or, more
significantly, recombinant ALVAC expressing the _env_
coding sequence from HIV-1 demonstrated V3-specific
cytotoxic responses.

20   **Example 7   -   CHARACTERIZATION OF CYTOTOXIC EFFECTOR
CELLS**

To determine the identity of the spleen cells
associated with the lysis of HIV-1 V3 peptide pulsed
target cells, mice were immunized with vCP112.  After

25   each immunization, or _in vitro_ stimulation 21 days after
the first inoculation, a two-step depletion procedure was
performed, and the spleen cells were assessed for
cytotoxicity against V3 peptide pulsed P815 cells.  Mice
inoculated with the canarypox vector ALVAC did not

30   generate spleen cells capable of killing peptide pulsed
targets.  Following a single immunization, vCP112 induced
spleen cells able to kill V3 peptide pulsed targets.  The
lytic effector cells were sensitive to treatment with
anti-murine Thy 1.2 or Lyt 2.2 plus complement and were

35   resistant to anti-CD4.  Fig. 10 shows the sensitivity of
the cytotoxic effector cells from spleen cells of mice
immunized with vCP112 to antibodies against cytotoxic T

67

lymphocyte cell surface antigens Thy 1.2 and Lyt 2.2.
Neither complement nor any of the monoclonal antibodies
or alloantisera alone affected the cytolytic action of
these cells. Similar results were obtained five days
5    after a second immunization administered on day 30.
Twenty-one days after a single inoculation, *in vitro*
stimulation with vCP112 infected syngeneic spleen cells
gave rise to lytic effector cells only partially
sensitive to anti-Thy 1.2 although completely sensitive
10   to anti-Lyt 2.2 and resistant to anti-CD4. These Thy
1.2-, CD4-, Lyt 2.2+ effector cells are not seen
following *in vitro* stimulation with vP911 of spleen cells
from vCP112 inoculated mice. Nonetheless, it is clear
that HIV V3 loop specific cytotoxicity was mediated by a
15   population of T lymphocytes expressing Thy 1.2 and Lyt
2.2, but not CD4. This cell surface phenotype is
characteristic of classical cytotoxic T lymphocytes.

**Example 8  -  SPECIFICITY OF CTL ANTIGEN RECEPTOR
                RECOGNITION OF THE V3 LOOP REGION OF HIV**
20   **              gp120**
         To examine the specificity of cytotoxic cells
generated by the HIV canarypox virus (ALVAC) recombinant,
vCP112 susceptibility to CTL activity was compared among
P815 target cells pulsed with peptides corresponding the
25   V3 loop region of gp120 of HIV isolates IIIB, MN, or SF2.
HIV specific primary CTL activity was confined only to
P815 target cells pulsed with peptide corresponding to
the V3 loop of HIV isolate IIIB, but not target cells
pulsed with peptides corresponding to the V3 loop region
30   of gp120 of HIV isolates MN or SF2, as shown in Fig. 8
which illustrates the specificity of cytotoxic T
lymphocyte antigen receptor for the HIV IIIB
hypervariable V3 loop of gp120, but not for the V3 loop
of HIV MN or SF2. Similar results were obtained with *in*
35   *vitro* stimulated, HIV specific memory CTL activity and
secondary CTL activity induced by immunization with the
ALVAC recombinant vCP112. Thus, HIV specific CTLs

68

elicited by a recombinant canarypox virus expressing the
env gene of HIV isolate IIIB recognize only target
epitopes derived from the same antigenic isolate. These
results clearly indicate the exquisite specificity of the
5  lymphocyte effector cells generated by immunization with
the HIV canarypox virus recombinant and eliminate such
nonspecific effector mechanisms as natural killer (NK)
cell activity.

     Example 9  -   ANTIBODY RESPONSES OF MICE INOCULATED WITH
10                  NYVAC- and ALVAC-BASED HIV RECOMBINANTS

          To evaluate humoral responses to HIV, mice were
immunized at day 0 with a NYVAC HIV recombinant or
canarypoxvirus (ALVAC) recombinant and received a
secondary immunization at week four.  The mice were bled
15 at various intervals through 20 weeks after the initial
immunization.  Pooled sera from each treatment group were
assayed for antibodies to HIV by ELISA employing purified
gp120 as antigen; the results are shown in Fig. 9 which
provides the antibody responses to HIV IIIB gp120 of mice
20 immunized with vectors (NYVAC, ALVAC) or with NYVAC
recombinant vP911 or ALVAC recombinant (vCP112)
expressing HIV-1 env, wherein the inverted triangle
indicates the time of administration of the second
inoculation.  Primary antibody responses were generally
25 modest, but detectable.  Following the secondary
immunization, the antibody titers of mice immunized with
both vP911 and vCP112 increased and peaked at week six
with titers of over 10,000.  These antibody titers
remained at approximately the same levels throughout the
30 duration of the study.  Thus, an ALVAC HIV recombinant,
vCP112, was capable of inducing a significant antibody
response.

          Inoculation of mice with ALVAC expressing the
env gene of HIV-1 elicits spleen cell reactivity with
35 characteristics of cytotoxic T lymphocytes: the
requirement for immunization, cell surface phenotype,
memory, and elegant epitope specificity.  Furthermore,

antibody responses to HIV-1 gp120 are induced by
inoculation with this ALVAC recombinant.

**Example 10  =  DERIVATION OF NYVAC- AND ALVAC-BASED HIV-1**
**RECOMBINANTS AND EXPRESSION OF HIV-1(MN)**
5                               **env BY ALVAC AND NYVAC**

          HIV-1(MN) env sequences were derived from
plasmid pMN1.8-9 and pMN1.8-10 which contain a 1774 bp
and 1803 bp subfragment from a genomic cDNA clone of HIV-
1(MN), respectively.  These plasmids were provided by the
10   laboratory of Dr. R.C. Gallo (NCI-NIH).  A 1,026 bp
KpnI/EcoRI fragment was derived by amplifying these
sequences from pMN1.8-9 by PCR using oligonucleotides
HIVMN6 (SEQ ID NO:82) (5'-GGGTTATTAATGATCTGTAG-3') and
HIV3B2 (SEQ ID NO:39) followed by digestion with
15   KpnI/EcoRI.  This fragment was inserted into pBS-SK
digested with KpnI and EcoRI to yield pBSMIDMN.

          A 1,028 bp SalI/XbaI fragment was derived from
pMN1.8-10 by PCR using oligonucleotides HIVMN5 (SEQ ID
NO:83) (5'-ATCATCGAGCTCTGTTCCTTGGGTTCTTAG-3') and HIVMN3P
20   (SEQ ID NO:84) (5'-
ATCATCTCTAGAATAAAAATTATAGCAAAGCCCTTTCCAAGCC-3') followed
by digestion with SacI and XbaI.  This fragment was co-
ligated into pBS-SK digested with EcoRI and XbaI with a
404 bp EcoRI/SacI fragment.  The 404 bp fragment was
25   derived by PCR with pMN1.8-9 as template and
oligonucleotides HIV3B1 (SEQ ID NO:32) and HIVMN4 (SEQ ID
NO:85) (5'-ATCATCGAGCTCCTATCGCTGCTC-3').  The resultant
plasmid was designated as pBS3MN.

          The 1,026 bp EcoRI/KpnI fragment from pBSMIDMN
30   was inserted into the 4,315 bp pBS3MN digested with
EcoRI/KpnI to generate pBSMID3MN.  This plasmid contains
most of the env gene except the 5'-most region.  The
vaccinia virus H6 promoter (Goebel et al., 1990a,b) and
the 5'-most region of the env gene were obtained by
35   isolating a 318 bp KpnI fragment from pBSH6HIV3B5P
(defined in Example 2).  This fragment was ligated into
KpnI/XbaI digested pBS-SK along with the 2.9 bp KpnI/XbaI

70

fragment from pBSMID3MN. The resultant plasmid was
designated as pH6HMNE.

   The 2.7 kb <u>Nru</u>I/<u>Xba</u>I fragment from pH6HMNE,
containing the entire HIV-1(MN) <u>env</u> gene juxtaposed 3' to
5  the 3'-most 26 bp of the H6 promoter, was blunt-ended and
inserted into <u>Nru</u>I/<u>Sma</u>I digested pSPHAH6. This generated
plasmid pHAHIVMNE. Plasmid pSPHAH6 was derived as
follows. Plasmid pMP2VCL (containing a polylinker region
within vaccinia sequences upstream of the K1L host range
10  gene) was digested within the polylinker with <u>Hind</u>III and
<u>Xho</u>I and ligated to annealed oligonucleotides SPHPRHA A
through D (SPHPRHA <u>A</u> (SEQ ID NO:86) 5'-
AGCTTCTTTATTCTATACTTAAAAAGTGAAAATAAATACAAAGGTTCTTGAGGGT -
3'

15  SPHPRHA <u>B</u> (SEQ ID NO:87) (5'-
TGTGTTAAATTGAAAGCGAGAAATAATCATAAATTATTTCATTATCGCGATATCCGT
TAAGTTTGTATCGTAC-3')

   SPHPRHA <u>C</u> (SEQ ID NO:88) (3'-
TTATTAGTATTTAATAAAGTAATAGCGCTATAGGCAATTCAAACATAGCATGAGCT-
20  5')

   SPHPRHA <u>D</u> (SEQ ID NO:89) (3'-
AGAAATAAGATATGAATTTTTCACTTTTATTTATGTTTCCAAGAACTCCCAACACAA
TTTAACTTTCGCTCT-5') generating pSP126 containing a
<u>Hind</u>III site, H6 promoter -124 through -1 (Perkus et al.,
25  1989) and <u>Xho</u>I, <u>Kpn</u>I, <u>Sma</u>I, <u>Sac</u>I and <u>Eco</u>RI sites.

   Plasmid pSD544 (containing vaccinia sequences
surrounding the site of the HA gene replaced with a
polylinker region and translation termination codons in
six reading frames) was digested with <u>Xho</u>I within the
30  polylinker, filled in with the Klenow fragment of DNA
polymerase I and treated with alkaline phosphatase.
SP126 was digested with <u>Hind</u>III, treated with Klenow and
the H6 promoter isolated by digestion with <u>Sma</u>I.
Ligation of the H6 promoter fragment to pSD544 generated
35  pSPHAH6 which contained the H6 promoter in the polylinker
region (in the direction of HA transcription). This
insertion plasmid enables the replacement of the vaccinia

71

HA gene (A56; Goebel et al., 1990a,b) with foreign
genetic material.

The C5L insertion plasmid was derived as
follows. Using the cosmid vector pVK102 (Knauf and
5   Nester, 1982), a genomic library for vCP65 (ALVAC-based
rabies G recombinant with rabies in C5 locus) was
constructed. This library was probed with the 0.9 kb
PvuII canarypoxvirus genomic fragment contained within
pRW764.5 (C5 locus). These canarypox DNA sequences
10  contain the original insertion locus. A clone containing
a 29 kb insert was grown up and designated pHCOS1. From
this cosmid containing C5 sequences, a 3.3 kb Cla
fragment was subcloned. Sequence analysis from this ClaI
fragment was used to extend the map of the C5 locus from
15  1-1372.

The C5 insertion vector, pC5L, was constructed
in two steps. The 1535 bp left arm was generated by PCR
amplification using oligonucleotides C5A (SEQ ID NO:90)
(5'-ATCATCGAATTCTGAATGTTAAATGTTATACTTTG-3') and C5B (SEQ
20  ID NO:91) (5'-GGGGGTACCTTTGAGAGTACCACTTCAG-3'). The
template DNA was canarypoxvirus genomic DNA. This
fragment was cloned into EcoRI/SmaI digested pUC8. The
sequence was confirmed by standard sequencing protocols.
The 404 bp right arm was generated by PCR amplification
25  using oligonucleotides C5C (SEQ ID NO:92) (5'-
ATCATCCTGCAGGTATTCTAAACTAGGAATAGATG-3') and C5DA (SEQ ID
NO:93) (5'-ATCATCCTGCAGGTATTCTAAACTAGGAATAGATG-3). This
fragment was then cloned into the vector previously
generated containing the left arm digested with
30  SmaI/PstI. The entire construct was confirmed by
standard sequence analysis and designated pC5L. This
insertion plasmid enables the insertion of foreign genes
into the C5 locus.

The 2.8 kb XbaI/partial KpnI fragment from
35  pH6HMNE was isolated and inserted into pC5L digested with
XbaI and KpnI. The resultant plasmid was designated as
pC5HIVMNE.

72

   Plasmids pHAHIVMNE and pC5HIVMNE were used *in vitro* recombination experiments with NYVAC (vP866) and ALVAC (CPpp), respectively, as the rescue virus.  These were done by standard procedures (Piccini et al., 1987).
5  Plaques derived from recombinant virus were identified by plaque hybridization using a radiolabeled env-specific DNA probe (Piccini et al., 1987).  After three rounds of plaque purification, the recombinant viruses were amplified.  The NYVAC-based HIV-1(MN) env recombinant was
10 designated vP1008 and the ALVAC-based recombinant vCP125.

   Recombinant viruses, vCP125 and vP1008, were analyzed for expression of the HIV-1(MN) env gene by immunofluorescence and immunoprecipitation using previously reported procedures (Taylor et al., 1990).
15 Pooled human sera from HIV-seropositive individuals (obtained from Dr. K. Steimer, Chiron Corp., Emeryville, CA) was used in these assays.  Results from immunofluorescence revealed that cells infected with either vCP125 or vP1008 express the HIV-1(MN) gene
20 product on their surface.  Immunoprecipitation from lysates prepared from vP1008 and vCP125 infected cells demonstrated the presence of three predominant HIV-1-specific proteins with apparent molecular masses of 160 kDa, 120 kDa, and 41 kDa, respectively.  These are
25 consistent with expression of the precursor envelope glycoprotein (160 kDa) and the proteolytically derived mature forms (120 kDa and 41 kDa).

   **Example 11  -  EXPRESSION OF THE HIV-1(MN) gp120**
   **BY NYVAC AND ALVAC**

30     A 391 bp EcoRI/XbaI fragment was amplified from pBS3MN using oligonucleotides T7 (SEQ ID NO:94) (5'-AATACGACTCACTATAG-3') and HIVMN120 (SEQ ID NO:95) (5'-ATCATCTCTAGAATAAAAATTATCTTTTTTTCTCTCTGCACCACTC-3') followed by digestion with EcoRI and XbaI.  This fragment
35 was ligated to the 4.2 kb EcoRI/XbaI fragment derived from pH6HMNE (defined in Example 10).  The resultant

73

plasmid contains a poxvirus expression cassette for HIV-
1(MN) gp120 in pBS-SK and was designated pBSHIVMN120.

A 1.7 kb XbaI/partial KpnI fragment was
isolated and inserted into pC5L digested with KpnI/XbaI.
5    The resultant plasmid was designated as pC5HIVMN120.  The
insertion plasmid for integrating the HIV-1(MN) gp120
gene into NYVAC was obtained by first isolating the 1.6
kb NruI/SmaI fragment from pBSHIVMN120.  This fragment
was inserted into pSPHAH6 digested with NruI and SmaI to
10   provide pHAHIVMN120.

Insertion plasmids, pC5HIVMN120 and
pHAHIVMN120, were used in recombination experiments with
ALVAC (CPpp) and NYVAC (vP866) as the rescuing virus.
These assays and plaque identification and purification
15   were performed by standard procedures (Piccini et al.,
1987).  Hybridization analyses were performed with a
radiolabeled HIV-1(MN) gp120-specific probe.  Purified
recombinants were amplified.  The ALVAC-based HIV-1(MN)
gp120 recombinant was designated as vCP124 and the NYVAC-
20   based HIV-1(MN) gp120 recombinant as vP1004.

Cells infected with vCP124 and vP1004 were
analyzed for the presence of the recombinant expressed
HIV-1(MN) gp120 by immunofluorescence and
immunoprecipitation.  These assays were performed as
25   previously described (Taylor et al., 1990) using a pooled
human sera from HIV-seropositive individuals (obtained
from K. Steimer, Chiron Corporation, Emeryville, CA).
Results from these studies clearly indicated that cells
infected with either vCP124 and vP1004 contained HIV-
30   1(MN) gp120, whereas gp120 was not observed in uninfected
cells and cells infected with parental viruses, ALVAC and
NYVAC.

Example 12    -    EXPRESSION OF A NON-CLEAVABLE FORM OF
                   HIV-1 gp160 BY ALVAC AND NYVAC

35          In order to express a non-cleavable form of the
HIV-1 (IIIB) gp160 an arginine to threonine mutation was
engineered at amino acid 511 (Ratner et al., 1985) as was

74

demonstrated by Guo et al. (1990). These modifications
were made to decrease the shedding of gp120 from the
surface of infected cells. These manipulations were
performed as follows. A 376 bp PstI/XbaI fragment was
5 obtained by first amplifying the sequences from
pBSHIV3BEII (described in Example 2) using
oligonucleotides HIV3B2A (SEQ ID NO:96) (5'-
GAAATAATAAAACAATAATC-3') and HIVECB (SEQ ID NO:97) (5'-
GCTCCTATTCCCACTGCAGTTTTTTCTCTCTGCAC-3') followed by
10 digestion with PstI and XbaI. This fragment was ligated
with a 1,061 bp PstI/XbaI fragment and a 4.5 kb
EcoRI/XbaI fragment from pBSHIV3BEII to yield
pBSHIV3BEEC.

    The central region of the Hantaan virus S
15 segment was generated by PCR using oligonucleotides
T5HT3PPS (SEQ ID NO:98)
(5'GTCCTGCAGGATGGAAAAGAATGCCCCAAGC-3') and HTS55PN (SEQ
ID NO:99) (5'-GGGGGAGGCAAACTACCAAGG-3') and the S⁻
specific cDNA clone as template. The 581 bp fragment
20 contains a PstI site at its 3' end and the 5' end
includes the NciI site of position 499 of the S segment
(Schmaljohn et al., 1986). Furthermore, using the
oligonucleotide T5HT3PPS (SEQ ID NO:98) eliminates the
$T_5NT$ element at positoin 1029 to 1035 without altering
25 the amino acid sequence. This fragment was then digested
with NciI and PstI. The PCR fragment containing the 5'
end of the coding sequence fused to the H6 promoter
(HindIII/NciI digested above) was ligated into pBS-SK
digested with HindIII and PstI along with the 581 bp
30 NciI/PstI fragment containing the central region of the S
segment. The resultant plasmid was designated
pBSHTSH65P.

    The 3' most 438 bp of the S segment was derived
by PCR using oligonucleotides HTS3PXBA (SEQ ID NO:100)
35 (5'-ATCATCTCTAGAATAAAAATTAGAGTTTCAAAGGC-3') and T5HT5PSP
(SEQ ID NO:101) (5'-CGCCAGCATGCAGAAGCAGC-3') and the S-
specific cDNA clone as template. The 5' end of this

fragment contains the PstI site situated at position 1039
of the S segment coding sequence (Schmaljohn et al.,
1986) and the 3' end contains a T5NT sequence motif and a
unique XbaI prior to insertion into PstI/XbaI digested
5  pBS-SK to yield pBSHTS3P.

To generate the entire S segment expression
cassette, a 1122 bp PstI/partial HindIII fragment was
derived from pBSHTSH56P.  This fragment was co-inserted
into HindIII/XbaI digested pBS-SK with a 290 bp PstI/XbaI
10  fragment from pBSHTS3P.  The resultant plasmid was
designated pBSHVS by linearization with XbaI followed by
a partial HindIII digestion.

The 2.6 kb NruI/XbaI fragment from pBSHIV3BEEC,
containing the 3'-most 26 bp of the H6 promoter linked to
15  the gp160 cassette, was isolated and ligated to a 3.0 kb
NruI/XbaI fragment of pBSHVS to yield pBSHIV3BEECM.
Digestion with NruI and XbaI excises the 3'-most 26 bp of
the H6 promoter and the Hantaan virus S sequence.  The
3.0 kb NruI/XbaI fragment contains the 5'-most 100 bp of
20  the H6 promoter in a pBS-SK plasmid.

The 2.8 kb XbaI/partial KpnI fragment from
pBSHIV3BEECM was ligated to XbaI/KpnI digested pC5L to
yield pC5HIV3BEEC.  A 2.7 kb NruI/XbaI fragment from
pBSHIV3BEECM was blunt-ended with the Klenow fragment of
25  the E. coli DNA polymerase and inserted into NruI/SmaI
digested pSPHAH6 to yield pHAHIV3BEEC.

The insertion plasmids, pC5HIV3BEEC and
pHAHIV3BEEC, were used in in vitro recombination
experiments by standard procedures (Piccini et al., 1987)
30  using ALVAC (CPpp) and NYVAC (vP866), respectively, as
rescue virus.  Recombinant plaques were identified by
standard plaque hybridization analysis (Piccini et al.,
1987) using a radiolabeled probe specific for the HIV-1
env gene.  Recombinant viruses were amplified following
35  three rounds of purification.  The recombinant ALVAC-
based HIV-1(IIIB) gp160 (non-cleavable) was designated as
vCP126 and the NYVAC-based equivalent as vP1020.

Immunofluorescence and immunoprecipitation
analyses were performed by previously described
procedures on vP1020 and vCP126 infected cells using
pooled human serum from HIV-seropositive individuals

5   (obtained from K. Steimer, Chiron Corp., Emeryville, CA).
Immunofluorescence results clearly demonstrated the
surface expression of the HIV-1(IIIB) gp160 (non-
cleavable form) on the surface of cells infected with
either vCP126 or vP1020. Furthermore,

10  immunoprecipitation results demonstrated the presence of
a HIV-1(IIIB) gp160 in these infected cells that was not
proteolytically cleaved into the mature gp120 and gp41
frames.

        A non-cleavable form of the HIV-1(MN) gp160 was

15  also pursued and the recombinant viruses obtained as
follows.

        A PstI/XbaI fragment was obtained by PCR
amplification from pH6HMNE (described in Example 10)
using oligonucleotides HIVMN3P (SEQ ID NO:102) (5'-

20  ATCATCTCTAGAATAAAAATTATAGGAAAGCCCTTTCCAAGCC-3') and
HIVECA (SEQ ID NO:103) (5'-
GTGCAGAGAAAAAACTGCAGTGGGAATAGGAGC-3') followed by
digestion with PstI and XbaI. This 1061 bp fragment was
ligated with the 391 bp EcoRI/PstI from pBSHIVMNT

25  (described in Example 13) and the 4.2 kb EcoRI/XbaI
fragment from pH6HMNE (defined in Example 10). The
resultant plasmid was designated as pBSHIVMNEEC[1].
Sequence analysis of the HIV env insert demonstrated that
a single nucleotide was missing (between the SacI and

30  XbaI sites). To correct this, the following
manipulations were performed. The 1,028 kb SacI/XbaI
from pH6HMNE was used to substitute for the corresponding
fragment from pBSHIVMNEEC resulting in the formation of
pBSHIVMNEEC.

35      The 2.6 NruI/XbaI fragment from pBSHIVMNEEC was
isolated, blunt-ended with Klenow, and inserted into
NruI/SmaI digested pSPHAH6 (defined in Example 10). The

77

resultant plasmid was designated pHAHIVMNEEC. The 2.6 kb
Nru̲I/X̲b̲a̲I fragment from pBSHIVMNEEC was also inserted
into Nru̲I/X̲b̲a̲I digested pVQH6C5LSP6 (below) to yield
pC5HIVMNEEC.

5              Insertion plasmids, pHAHIVMNEEC and
pC5HIVMNEEC, were used in standard recombination
experiments (Piccini et al., 1987) with NYVAC (vP866) and
ALVAC (CPpp), respectively, as rescue virus. Recombinant
virus was identified and plaque purified by standard
10   plaque hybridization (Piccini et al., 1987) using a
radiolabeled HIV e̲n̲v̲-specific DNA probe. Purified
recombinant virus were then amplified. The NYVAC-based
recombinant containing the HIV-1 (MN) non-cleavable gp160
was designated as vP1078 and the ALVAC equivalent vCP144.

15              Expression analysis of vCP126 and vP1078 was
performed as described above. These results demonstrated
that expression was qualitatively equivalent to the HIV-1
(IIIB) counterparts, vP1020 and vCP126.

**Example 13    -  EXPRESSION OF A NON-CLEAVABLE, SECRETED**
20                 **FORM OF HIV-1 env BY ALVAC AND NYVAC**
              ALVAC- and NYVAC-based recombinant viruses were
generated which express an HIV-1(MN) e̲n̲v̲ that is not
proteolytically cleaved and is secreted by virtue of the
elimination of the transmembrane sequence near the
25   carboxy terminus of the gene product. A 502 bp Ps̲t̲I/X̲b̲a̲I
fragment was obtained by first amplifying these sequences
from pH6HMNE (defined in Example 10) using
oligonucleotides HIVECA (SEQ ID NO:103) and HIVMNT1 (SEQ
ID NO:104) (5'-
30   ATCATCTCTAGAATAAAAATTACAAACTTGCCCATTTATCCAATTCC-3')
followed by digestion with Ps̲t̲I (5'-end) and X̲b̲a̲I (3'-
end). This fragment corresponds to nucleotides 7219 to
7808 (Ratner et al., 1985). This fragment will serve as
the 3'-end of the e̲n̲v̲ expression cassette. As such, the
35   e̲n̲v̲ gene product will lack the transmembrane region, will
be terminated by a termination codon provided by
oligonucleotide HIVMNT1 (SEQ ID NO:104), and will not be

cleaved due to an amino change at 511 (defined in Example
12) provided using oligonucleotide HIVECA (SEQ ID
NO:103). This 502 bp fragment was ligated to the 391 bp
EcoRI/PstI fragment derived by PCR from pH6HMNE using
5   oligonucleotides HIV3B1 (SEQ ID NO:32) and HIVECB (SEQ ID
NO:97), and the 4.2 kb EcoRI/XbaI fragment to pH6MNE.
The resultant plasmid was designated pBSHIVMNT.

The 2.2 kb XbaI/partial KpnI fragment from
pBSHIVMNT was isolated and inserted into pC5L digested
10  with XbaI and KpnI. The resultant plasmid was designated
as pC5HIVMNT. The NYVAC insertion plasmid was derived by
isolating the 2.1 kb NruI/XbaI fragment from pBSHIVMNT.
This fragment was then blunt-ended with the Klenow
fragment of the E. coli DNA polymerase in the presence of
15  2mM dNTPs and inserted into pSPHAH6 digested with NruI
and SmaI to yield pHAHIVMNT.

The insertion plasmids, pC5HIVMNT and
pHAHIVMNT, were used in standard recombination
experiments (Piccini et al., 1987) with ALVAC (CPpp) and
20  NYVAC (vP866), respectively, as the rescue virus.
Recombinant virus was identified by standard plaque
hybridization assays (Piccini et al., 1987) using a
radiolabeled HIV env-specific probe. Recombinant virus
was subjected to three rounds of purification prior to
25  amplification. The ALVAC-based HIV-1(MN) env (non-
cleavable; secreted) was designated as vCP120 and the
NYVAC equivalent as vP994.

Immunoprecipitation analyses were performed as
previously described (above) for vCP120 and vP994
30  infected cells using pooled human sera from HIV-
seropositive individuals. Both vCP120 and vP994
expressed an HIV-1(MN) env-specific gene product with a
molecular weight consistent with a non-cleavable,
truncated gene product. Furthermore, immunoprecipitation
35  of the cell-free medium from vCP120 and vP994 infected
cell cultures indicated the secretion of this env gene
product.

A similar construction was engineered for the
HIV-1(IIIB) env. The following manipulations were
performed to accomplish this. A 487 bp PstI/XbaI
fragment was obtained by first amplifying these sequences
5 from pBSH6HIV3B5P (defined in Example 2) using
oligonucleotides HIVECA (SEQ ID NO:103) and HIV3BT (SEQ
ID NO:105) (5'-
ATCATCTCTAGAATAAAAATTACAAACTTGCCCATTTATCTAATTCC-3')
followed by digestion with PstI and XbaI. A 397 bp
10 EcoRI/PstI fragment was isolated from pBSHIV3BEEC and a
4.2 kb EcoRI/XbaI fragment was isolated from pH6HIIIBEM.
These three fragments were ligated together to yield
pBSHIV3BT1. Plasmid pH6HIIIBEM was derived from
pBSHIV3BEII (defined in Example 2) by digestion with KpnI
15 to liberate a second copy of the H6 promoter linked to
the 5' portion of the HIV-1 (IIIB) env gene. The 5.4 kp
KpnI was then religated to form pBSHIV3BEII.
        The 2.1 kb and 2.9 kb fragments derived by
HindIII/XbaI digestion of pBSHIV3BEECM were ligated to
20 the 105 bp HindIII/XbaI fragment from pBSHIV3BT1 to yield
pBSHIV3BT. This plasmid was digested with NruI and XbaI
to excise a 2.1 kb fragment. This fragment was blunt-
ended and inserted into pSPHAH6 digested with NruI and
SmaI to generate pHAHIV3BT.
25        The plasmid pHAHIV3BT was used in recombination
experiments, as above, with NYVAC (vP866) as the rescue
virus. Recombinant virus was identified and purified as
above and the resultant recombinant was designated as
vP1036. This recombinant had all the expression
30 characteristics noted above for vCP120 and vP994.
        Example 14 - **EXPRESSION OF HIV-1(MN) gp120 ANCHORED
                     WITH A TRANSMEMBRANE SEQUENCE BY NYVAC AND
                     ALVAC**
        To fuse the env region encoding the gp120 to
35 the region encoding the hydrophobic transmembrane
sequence, the following manipulations were performed. A
200 bp fragment corresponding to the 3'-most region of

80

the gp120 coding sequence was derived by PCR from pH6HMNE
(defined in Example 10) using oligonucleotides HIV3B1
(SEQ ID NO:32) and HIVMN18 (SEQ ID NO:106) (5'-
GCCTCCTACTATCATTATGAATAATCTTTTTCTCTCTG-3'). This

5    fragment was fused by PCR to annealed oligonucleotides
HIVTM1 (SEQ ID NO:107) (5'-
TTATTCATAATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTC
TCTGTAGTGAATAGAGTTAGGCAGGGATAA-3') and HIVTM2 (SEQ ID
NO:108) (5'-

10   TTATCCCTGCCTAACTCTATTCACTACAGAGAGTACAGCAAAAACTATTCTTAAACC
TACCAAGCCTCCTACTATCATTATGAATAA-3') using oligonucleotides
HIV3B1 (SEQ ID NO:32) and HIVTM3 (SEQ ID NO:109) (5'-
ATCATCTCTAGAATAAAAATTATCCCTGCCTAACTCTATTCAC-3').
Oligonucleotides HIVTM1 (SEQ ID NO:107) and HIVTM2 (SEQ

15   ID NO:108) correspond to nucleotides 7850 to 7934 (Ratner
et al., 1985) and represent the region encoding the HIV
env hydrophobic anchor sequence. Fusion with HIVTM3 (SEQ
ID NO:109) engineers the 3'-end of the eventual cassette
with a termination codon and a 3' XbaI site. The derived

20   fragment was digested with EcoRI/XbaI and ligated to
pH6HMNE digested with EcoRI and XbaI to yield
pBSHIVMN120T.
     The 1.7 bk NruI/XbaI fragment from
pBSHIVMN120T, containing the 3'-most 26 bp of the H6

25   promoter and the entire HIV-1 cassette, was isolated and
inserted into the 5.1 kb NruI/XbaI fragment from
pVQH6C5LSP6 to derive pC5HIVMN120T. The plasmid
pVQH6C5LSP6 was derived as follows.
     pC5L (defined in Example 10) was digested

30   within the polylinker with Asp718 and NotI, treated with
alkaline phosphatase and ligated to kinased and annealed
oligonucleotides CP26 (SEQ ID NO:110) (5'-
GTACGTGACTAATTAGCTATAAAAAGGATCCGGTACCCTCGAGTCTAGAATCGATCC
CGGGTTTTTATGACTAGTTAATCAC-3') and CP27 (SEQ ID NO:111)

35   (5'-
GGCCGTGATTAACTAGTCATAAAAACCCGGGATCGATTCTAGACTCGAGGGTACCGG
ATCCTTTTTATAGCTAATTAGTCAC-3') (containing a disabled

Asp718 site, translation stop codons in six reading
frames, vaccinia early transcription termination signal
(Yuen and Moss, 1987), BamHI, KpnI, XhoI, XbaI, ClaI, and
SmaI restriction sites, vaccinia early transcription

5   termination signal, translation stop codons in six
reading frames, and a disabled NotI site) generating
plasmid pC5LSP.

pC5LSP was digested with BamHI and ligated to
annealed oligonucleotides CP32 (SEQ ID NO:112) (5'-

10  GATCTTAATTAATTAGTCATCAGGCAGGGCGAGAACGAGACTATCTGCTCGTTAATT
AATTAGGTCGACG-3') and CP33 (SEQ ID NO:113) (5'-
GATCCGTCGACCTAATTAATTAACGAGCACATAGTCTCGTTCTCGCCCTGCCTGATG
ACTAATTAATTAA-3') to generate pVQC5LSP6.

The 1.7 kb NruI/XbaI fragment from pBSHIVMN120T

15  was also blunt-ended and inserted into pSPHAH6 digested
with NruI and SmaI. The resultant plasmid was designated
as pHAHIVMN120T.

Insertion plasmids, pC5HIVMN120T and
pHAHIVMN120T, were used in standard recombination

20  experiments (Piccini et al., 1987) with ALVAC and NYVAC,
respectively, as the rescue virus. Recombinant virus was
identified and purified by standard plaque hybridization
(Piccini et al., 1987) using a radiolabeled HIV-1 gp120-
specific DNA probe. The pure populations were amplified

25  and the ALVAC-based anchored HIV-1(MN) gp120 recombinant
was designated vCP138. The NYVAC-based equivalent was
designated vP1035.

Immunofluorescence and immunoprecipitation
analyses were performed by standard procedures (above) to

30  evaluate expression of the HIV-1(MN) anchored gp120 in
vP138 and vP1035 infected cells. The assays were
performed using pooled human sera from HIV-seropositive
individuals (obtained from Dr. K. Steimer, Chiron Corp.,
Emeryville, CA). Investigation of surface

35  immunofluorescence indicated that vCP138 and vP1035
infected cells contained HIV-1(MN) gp120 in the plasma
membrane. Significantly, the surface staining of vCP138

and vP1035 infected cells was enhanced compared to cells
infected with recombinant viruses (i.e. vCP125, vCP124,
vP1004, and vP1008) expressing gp160 or a non-anchored
gp120.  Results from immunoprecipitation analyses

5    confirmed the expression of gp120 in vCP138 and vP1035
infected cells and that the expressed product was of the
expected molecular mass.

**Example 15  -  GENERATION OF NYVAC/HIV-1 GAG (PROTEASE⁻)**
                    **RECOMBINANT**

10             Plasmid pSD542 (a NYVAC TK locus donor plasmid;
see Example 5) was derived from plasmid pSD460 (Tartaglia
et al., 1992) by forming vector plasmids pSD513 as
described above in Example 7.  The polylinker region in
pSD513 was modified by cutting with PstI/BamHI and

15   ligating to annealed synthetic oligonucleotides MPSYN288
(SEQ ID NO:114) (5'GGTCGACGGATCCT 3') and MPSYN289 (SEQ
ID NO:115) (5' GATCAGGATCCGTCGACCTGCA 3') resulting in
plasmid pSD542.

               A plasmid, pHXB2D, containing human

20   immunodeficiency virus type 1 (HIV-1) cDNA sequence was
obtained from Dr. R.C. Gallo (NCI-NIH).  The sequence
encoding the 5'-end of the gag gene was cloned between
vaccinia virus tk flanking arms.  This was accomplished
by cloning the 1,625 bp BglII fragment of pHXB2D,

25   containing the 5'-end of the gag gene, into the 4,075 bp
BglII fragment of pSD542.  The plasmid generated by this
manipulation is called pHIVG2.

               The 3'-end of the gag gene was then cloned
downstream from the rest of the gag gene.  This was

30   accomplished by cloning a 280 bp ApaI-BamHI PCR fragment,
containing the 3'-end of the gag gene, into the 5,620 bp
ApaI-BamHI fragment of pHIVG2.  This PCR fragment was
generated from the plasmid, pHXB2D, with the
oligonucleotides, HIVP5 (SEQ ID NO:116) (5'-

35   TGTGGCAAAGAAGGGC-3') and HIVP6 (SEQ ID NO:117) (5'-
TTGGATCCTTATTGTGACGAGGGGTC-3').  The plasmid generated by
this manipulation is called pHIVG3.

The I3L promoter was then cloned upstream of
the gag gene. This was accomplished by cloning the
oligonucleotides, HIVL17 (SEQ ID NO:118) (5'-
GATCTTGAGATAAAGTGAAAATATATATCATTATATTACAAAGTACAATTATTTAGG
5    TTTAATCATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGAT-3') and
HIVL18 (SEQ ID NO:119) (5'-
CGATCTAATTCTCCCCCGCTTAATACTGACGCTCTCGCACCCATGATTAAACCTAAA
TAATTGTACTTTGTAATATAATGATATATATTTTCACTTTATCTCAA-3'),
encoding the vaccinia virus I3L promoter and the 5'-end
10   of the gag gene, into the 5,540 bp partial BglII-ClaI
fragment of pHIVG3. The plasmid generated by this
manipulation is called pHIVG4.

        pHIVG4 was used in recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP969.
15       Immunoprecipitation analysis was performed to
determine whether vP969 expresses authentic HIV-1 gag
precursor protein.

        Lysates from the infected cells were analyzed
for HIV-1 gag precursor expression using pooled serum
20   from HIV-1 seropositive individuals (obtained from
Chiron, Emeryville, CA). The sera was preadsorbed with
vP866 infected Vero cells. The preadsorbed sera was
bound to Protein A-sepharose in an overnight incubation
at 4°C. Following this incubation period, the material
25   was washed 4X with 1X buffer A. Lysates precleared with
normal human sera and protein A-sepharose were then
incubated overnight at 4°C with the HIV-1 seropositive
human sera bound to protein A-sepharose. After the
overnight incubation period, the samples were washed 4X
30   with 1X buffer A and 2X with a LiCl$_2$/urea buffer.
Precipitated proteins were dissociated from the immune
complexes by the addition of 2X Laemmli's buffer (125 mM
Tris (pH6.8), 4% SDS, 20% glycerol, 10% 2-
mercaptoethanol) and boiling for 5 min. Proteins were
35   fractionated on a 10% Dreyfuss gel system (Dreyfuss et
al., 1984), fixed and treated with 1M Na-salicylate for
fluorography.

Human sera from HIV-1 seropositive individuals specifically precipitated the HIV-1 _gag_ precursor protein from vP969 infected cells, but did not precipitate HIV-1-specific proteins from mock infected or NYVAC infected

5  cells.

Example 16  -  GENERATION OF NYVAC/HIV-1 _gag/pol_
                  RECOMBINANT

The sequence encoding the 5'-end of the _gag_ gene was cloned between vaccinia virus tk flanking arms.

10 This was accomplished by cloning the 1,625 bp BglII fragment of pHXB2D, containing the 5'-end of the _gag_ gene, into the 4,075 bp BglII fragment of pSD542 (defined in Example 15). The plasmid generated by this manipulation is called pHIVG2.

15      The 3'-end of the _gag_ gene was then cloned into pHIVG2. This was accomplished by cloning a 280 bp ApaI-BamHI PCR fragment, containing the 3'-end of the _gag_ gene, into the 5,620 bp ApaI-BamHI fragment of pHIVG2. This PCR fragment was generated from the plasmid, pHXB2D,

20 with the oligonucleotides, HIVP5 (SEQ ID NO:116) and HIVP6 (SEQ ID NO:117). The plasmid generated by this manipulation is called pHIVG3.

The I3L promoter was then cloned upstream of the _gag_ gene. This was accomplished by cloning the

25 oligonucleotides, HIVL17 (SEQ ID NO:118) and HIVL18 (SEQ ID NO:119), encoding the vaccinia virus I3L promoter and the 5'-end of the _gag_ gene, into the 5,540 bp partial BglII-ClaI fragment of pHIVG3. The plasmid generated by this manipulation is called pHIVG4.

30      The portion of the _gag_ gene encoding p24, p2, p7 and p6 was then eliminated. This was accomplished by cloning the oligonucleotides, HIVL19 (SEQ ID NO:120) (5'-CTGACACAGGACACAGCAATCAGGTCAGCCAAAATTACTAATTTTTATCTCGAGGTC GACAGGACCCG-3') and HIVL20 (SEQ ID NO:121) (5'-

35 GATCCGGGTCCTGTCGACCTCGAGATAAAAATTAGTAATTTTGGCTGACCTGATTGC TGTGTCCTGTGTCAG-3'), into the 4,450 bp partial PvuII-

<u>Bam</u>HI fragment of pHIVG4. The plasmid generated by this manipulation is called pHIVG5.

The remainder of the <u>gag</u> gene, as well as the <u>pol</u> gene, was then cloned downstream of the p17 "gene".
5   This was accomplished by cloning the 4,955 bp <u>Cla</u>I-<u>Sal</u>I fragment of pHXB2D, containing most of the <u>gag</u> gene and all of the <u>pol</u> gene, into the 4,150 bp <u>Cla</u>I-<u>Sal</u>I fragment of pHIVG5. The plasmid generated by this manipulation is called pHIVG6.

10   Extraneous 3'-noncoding sequence was then eliminated. This was accomplished by cloning a 360 bp <u>Afl</u>II-<u>Bam</u>HI PCR fragment, containing the 3'-end of the <u>pol</u> gene, into the 8,030 bp AflII-<u>Bam</u>HI fragment of pHIVG6. This PCR fragment was generated from the
15   plasmid, pHXB2D, with the oligonucleotides, HIVP7 (SEQ ID NO:122) (5'-AAGAAAATTATAGGAC-3') and HIVP8 (SEQ ID NO:123) (5'-TTGGATCCCTAATCCTCATCCTGT-3'). The plasmid generated by this manipulation is called pHIVG7.

pHIVG7 was used in recombination experiments
20   with vP866 (NYVAC) as the rescuing virus to yield vP989.

Immunoprecipitation experiments with vP989 infected cells were performed as described above for the expression of the HIV-1 <u>gag</u> precursor protein. No HIV-1-specific species were precipitated from mock infected or
25   NYVAC infected Vero cells. Protein species corresponding to the <u>gag</u> precursor protein, as well as various intermediate and mature <u>gag</u> cleavage products, were precipitated, however, from lysates of vP989 infected cells.

30   <u>**Example 17**</u>   -   **GENERATION OF NYVAC/HIV-1 gag/pol AND**
                     <u>**env (gp120) RECOMBINANT**</u>

The sequence encoding the 5'-end of the <u>gag</u> gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid
35   pHIVG7, as described above (<u>see</u> Example 16).

pHIVG7 was used in recombination experiments with vP921 as the rescuing virus to yield vP991.

86

Immunoprecipitation experiments with vP991
infected cells were performed as described above for the
expression of the HIV gag precursor protein.  No HIV-
specific species were precipitated from mock infected
5   Vero cells.  Protein species corresponding to the env and
gag precursor proteins, as well as various intermediate
and mature gag cleavage products, were precipitated,
however, from lysates of vP991 infected cells.
Example 18  -  GENERATION OF NYVAC/HIV-1 gag/pol AND
10                  env (gp160) RECOMBINANT
The sequence encoding the 5'-end of the gag
gene was cloned between vaccinia virus tk flanking arms.
This was accomplished by initially preparing plasmid
pHIVG7, as described above (see Example 16).
15          pHIVG7 was used in recombination experiments
with vP911 (above) as the rescuing virus to yield vP990.
Immunoprecipitation experiments with vP990
infected cells were performed as described above for the
expression of the HIV-1 gag precursor protein.  No HIV-1-
20  specific species were precipitated from mock infected
Vero cells.  Protein species corresponding to the env and
gag precursor proteins, as well as various intermediate
and mature gag cleavage products, were precipitated,
however, from lysates of vP990 infected cells.
25  Example 19  -  GENERATION OF NYVAC/HIV-1 p17, p24
                  RECOMBINANT
A plasmid, pHXB2D, containing HIV-1 cDNA
sequence, was obtained from Dr. R.C. Gallo (NCI-NIH).
The sequence encoding the 5'-end of the gag gene was
30  cloned between vaccinia virus tk flanking arms.  This was
accomplished by initially preparing pHIVG5, as described
above (see Example 16).
The 3'-end of the p24 "gene" was then cloned
into pHIVG5.  This was accomplished by cloning a 660 bp
35  SalI-BamHI PCR fragment, containing the 3'-end of the p24
"gene", into the 4,450 bp SalI-BamHI fragment of pHIVG5.
This PCR fragment was generated from the plasmid, pHXB2D,

with the oligonucleotides, HIVP25 (SEQ ID NO:124) (5'-
AAAGTCGACCCATATCACCTAGAAC-3') and HIVP26 (SEQ ID NO:125)
(5'-TTTGGATCCTTACAAAACTCTTGCCTTAT-3'). The plasmid
generated by this manipulation is called pHIVG8.

5          The entomopox 42 kd promoter was then cloned
upstream of the p24 "gene". This was accomplished by
cloning the oligonucleotides, HIVL21 (SEQ ID NO:126) (5'-
TCGAGCAAAATTGAAAATATATAATTACAATATAAAATGCCTATAGTGCAGAACATC
CAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCA-3') and
10   HIVL22 (SEQ ID NO:127) (5'-
TTTAAAGTTCTAGGTGATATGGCCTGATGTACCATTTGCCCCTGGATGTTCTGCACT
ATAGGCATTTTATATTGTAATTATATATTTTCAATTTTGC-3'), encoding
the entomopox 42 kd promoter and the 5'-end of the p24
"gene", into the 5,070 bp XhoI-NsiI fragment of pHIVG8.
15   The plasmid generated by this manipulation is called
pHIVG9.
          pHIVG9 was used in recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP970.
          Immunoprecipitation experiments with vP970
20   infected cells were performed as described above for the
expression of the HIV-1 gag precursor protein. No HIV-1-
specific species were precipitated from mock infected or
NYVAC infected Vero cells. A protein species
corresponding to p24 was precipitated, however, from
25   lysates of vP970 infected cells.
     Example 20  -  GENERATION OF NYVAC/HIV-1 p17, p24 AND
                     env (gp120) RECOMBINANT
          A plasmid, pHXB2D, containing HIV-1 cDNA
sequence, was obtained from Dr. R.C. Gallo (NCI-NIH).
30   The sequence encoding the 5'-end of the gag gene was
cloned between vaccinia virus tk flanking arms. This was
accomplished by initially preparing plasmid pHIVG9 as
described above (see Example 19).
          pHIVG9 was used in recombination experiments
35   with vP921 as the rescuing virus to yield vP973.
          Immunoprecipitation experiments with vP973
infected cells were performed as described above for the

88

expression of the HIV-1 gag precursor protein. No HIV-1-
specific species were precipitated from mock infected
Vero cells. Protein species corresponding to env and p24
were precipitated, however, from lysates of vP973
5   infected cells.

Example 21   -   GENERATION OF NYVAC/HIV-1 p17, p24 AND
               env (gp160) RECOMBINANT

        A plasmid, pHXB2D, containing HIV-1 cDNA
sequence, was obtained from Dr. R.C. Gallo (NCI-NIH).
10  The sequence encoding the 5'-end of the gag gene was
cloned between vaccinia virus tk flanking arms. This was
accomplished by initially preparing plasmid pHIVG9, as
described above (see Example 19).

        pHIVG9 was used in recombination experiments
15  with vP911 as the rescuing virus to yield vP971.

        Immunoprecipitation experiments with vP971
infected cells were performed as described above for the
expression of the HIV-1 gag precursor protein. No HIV-1-
specific species were precipitated from mock infected
20  Vero cells. Protein species corresponding to env and p24
were precipitated, however, from lysates of vP971
infected cells.

Example 22   -   GENERATION OF NYVAC/HIV-1 gag (PROTEASE⁻)
               AND env (TRUNCATED) RECOMBINANT

25      A plasmid, pHXB2D, containing HIV-1 cDNA
sequence was obtained from Dr. R.C. Gallo (NCI-NIH). The
sequence encoding the 5'-end of the gag gene was cloned
between vaccinia virus tk flanking arms. This was
accomplished by initially preparing plasmid pHIVG9 as
30  described above (see Example 19).

        An H6-promoted truncated HIV-1 envelope gene
was then inserted into pHIVG4. This was accomplished by
cloning the E. coli DNA polymerase I (Klenow fragment)
filled-in 1,600 bp XhoI-NotI fragment of pHIVE10,
35  containing an H6-promoted truncated HIV-1 envelope gene,
into the filled-in BamHI site of pHIVG4. The plasmid
generated by this manipulation is called pHIVGE11.

89

The plasmid pHIVE10 was derived by inserting a
SacI/partial KpnI fragment from pBSHIV3BCDT1 into the
multiple cloning region of pIBI25 (IBI, New Haven, CT).
The plasmid pBSHIV3BCDT1 contains an H6 promoted cassette
5    to express a severely truncated form of the HIV-1(IIIB)
envelope (amino acid 1 to 447; Ratner et al., 1985).
Expression of this cassette was evaluated to eliminate
CD4 binding while retaining the V3 loop region and the T1
epitope.

10           To construct pBSHIV3BCDT1 the following
manipulations were performed.  A PCR-derived fragment of
200 bp was amplified from pBSH6HIV3B5P (defined in
Example 2) using oligonucleotides HIV3B2A (SEQ ID NO:96)
and HIVCD4A (SEQ ID NO:128) (5'-
15   GCCTCCTACTATCATTATGAATAAACTGATGGGAGGGGCATAC-3').  This
fragment was fused by PCR to annealed oligonucleotides
HIVTM1 (SEQ ID NO:107) and HIVTM2 (SEQ ID NO:108) using
oligonucleotides HIV3B2A (SEQ ID NO:96) and HIVTM3 (SEQ
ID NO:109).  These manipulations create the 3'-end of the
20   truncated env cassette by placing sequences encoding the
HIV-1 env transmembrane anchor (amino acids 691 to 718;
Ratner et al., 1985), a translation termination codon
(TAA), and a 3' XbaI site.  This PCR-fusion product was
digested with EcoRI and XbaI to yield a 243 bp fragment.
25   The fragment was ligated to the 4.5 bp EcoRI/XbaI
fragment of pH6HIIIBE to generate pBSHIV3BCDT1.
             pHIVGE11 was used in recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP979.
             Immunoprecipitation experiments with vP979
30   infected cells were performed as described above for the
expression of the HIV-1 gag precursor protein.  No HIV-1-
specific species were precipitated from mock infected or
NYVAC infected Vero cells.  Protein species corresponding
to env and the gag precursor proteins were precipitated,
35   however, from lysates of vP979 infected cells.

**Example 23** - GENERATION OF NYVAC/HIV-1 gag/pol AND
               env (TRUNCATED) RECOMBINANT

        The sequence encoding the 5'-end of the gag
gene was cloned between vaccinia virus tk flanking arms.
5  This was accomplished by initially preparing plasmid
pHIVG7 as described above (see Example 16).

        An H6-promoted truncated HIV-1 envelope gene
was then inserted into pHIVG7. This was accomplished by
cloning the E. coli DNA polymerase I (Klenow fragment)
10  filled-in 1,600 bp XhoI-NotI fragment of pHIVE10 (defined
in Example 22), containing an H6-promoted truncated HIV-1
envelope gene, into the filled-in BamHI site of pHIVG7.
The plasmid generated by this manipulation is called
pHIVGE12.

15        pHIVGE12 was used in recombination experiments
with vP866 (NYVAC) as the rescuing virus to yield vP978.

        Immunoprecipitation experiments with vP978
infected cells were performed as described above for the
expression of the HIV-1 gag precursor protein. No HIV-1-
20  specific species were precipitated from mock infected or
NYVAC infected Vero cells. Protein species corresponding
to the env and gag precursor proteins, as well as various
intermediate and mature gag cleavage products, were
precipitated, however, from lysates of vP978 infected
25  cells.

**Example 24** - GENERATION OF NYVAC/HIV-1 gag/pol AND
               env (gp120) RECOMBINANT

        The sequence encoding the gag gene was cloned
between vaccinia virus tk flanking arms. This was
30  accomplished by initially preparing plasmid pHIVG7, as
described above (see Example 16).

        The I3L-promoted gag and pol genes were then
inserted into a canary pox insertion vector. This was
accomplished by cloning the 4,360 bp partial BglII-BamHI
35  fragment of pHIVG7, containing the I3L-promoted gag and
pol genes, into the BamHI site of pVQH6CP3L. The plasmid
generated by this manipulation is called pHIVGE14.

91

The H6-promoted HIV-1(MN) envelope (gp120) gene
was then inserted into pHIVGE14. This was accomplished
by cloning the oligonucleotides, HIVL29 (SEQ ID NO:129)
(5'-GGCCGCAAC-3') and HIVL30 (SEQ ID NO:130) (5'-
5    TCGAGTTGC-3'), and the 1,600 bp NruI-NotI fragment of
pBSHIVMN120, containing the H6-promoted gp120 gene, into
the 11,500 bp NruI-XhoI fragment of pHIVGE14. The
plasmid generated by this manipulation is called
pHIVGE15.

10           The H6-promoted envelope (gp120) gene and the
I3L-promoted gag and pol genes were then inserted into a
vaccinia virus insertion vector. This was accomplished
by cloning the 6,400 bp NruI-BamHI fragment of pHIVGE15,
containing the H6-promoted gp120 gene and the I3L-
15   promoted gag and pol genes, into the 4,000 bp NotI-BglII
fragment of pSD542VCVQ. The plasmid generated by this
manipulation is called pHIVGE16.

pHIVGE16 was used in in vitro recombination
experiments with vP866 (NYVAC) as the rescuing virus to
20   yield vP988.

Immunoprecipitation experiments with vP988
infected cells were performed as described above for the
expression of the HIV-1 gag precursor protein. No HIV-1-
specific species were precipitated from mock infected or
25   NYVAC infected Vero cells. Protein species corresponding
to the env and gag precursor proteins, as well as various
intermediate and mature gag cleavage products, were
precipitated, however, from lysates of vP988 infected
cells.

30   Example 25   -   GENERATION OF NYVAC/HIV-1 gag/pol AND
                        env (gp160) RECOMBINANT

The sequence encoding the 5'-end of the gag
gene was cloned between vaccinia virus tk flanking arms.
This was accomplished by initially preparing plasmid
35   pHIVGE16 as described above (see Example 24).

The gp120 gene was then replaced by the gp160
gene. This was accomplished by cloning the 2,600 bp

NruI-NotI fragment of pH6HMNE, containing the entire HIV-1(MN) envelope (gp160) gene, into the 8,000 bp partial NruI-NotI fragment of pHIVGE16. The plasmid generated by this manipulation is called pHIVGE19.

5      pHIVGE19 was used in *in vitro* recombination experiments with vP866 (NYVAC) as the rescuing virus to yield vP1009.

Immunoprecipitation experiments with vP1009 infected cells were performed as described above for the
10 expression of the HIV-1 gag precursor protein. No HIV-1-specific species were precipitated from mock infected or NYVAC infected Vero cells. Protein species corresponding to the env and gag precursor proteins, as well as various intermediate and mature gag cleavage products, were
15 precipitated, however, from lysates of vP1009 infected cells.

**Example 26  -  GENERATION OF ALVAC/HIV-1 gag/pol AND env (GP120) RECOMBINANT**

The sequence encoding the 5'-end of the gag
20 gene was cloned between vaccinia virus tk flanking arms. This was accomplished by initially preparing plasmid pHIVGE15, as described above (see Example 24).

pHIVGE15 was used in recombination experiments with ALVAC (CPpp) as the rescuing virus to yield vCP117.
25      Immunoprecipitation analysis was performed as described above but with CEF cell monolayers to determine whether vCP117 expresses authentic HIV-1 gag and env gene products.

Lysates from the infected cells were analyzed
30 for HIV gag and env gene expression using serum from HIV-1 seropositive individuals (obtained from New York State Department of Health). The sera was preadsorbed with ALVAC infected CEF cells. The preadsorbed sera was bound to Protein A-sepharose in an overnight incubation at 4°C.
35 Following this incubation period, the material was washed 4X with 1X buffer A. Lysates precleared with normal human sera and protein A-sepharose were then incubated

overnight at 4°C with the HIV-1 seropositive human sera
bound to protein A-sepharose. After the overnight
incubation period, the samples were washed 4X with 1X
buffer A and 2X with a LiCl$_2$/urea buffer. Precipitated
5    proteins were dissociated from the immune complexes by
the addition of 2X Laemmli's buffer (125 mM Tris (pH6.8),
4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling
for 5 min. Proteins were fractionated on a 10% Dreyfuss
gel system (Dreyfuss et al., 1984), fixed and treated
10   with 1M Na-salicylate for fluorography.

        Human sera from HIV-1 seropositive individuals
specifically precipitated the HIV-1 gag and env proteins
from vCP117 infected cells, but did not precipitate HIV-
1-specific proteins from mock infected or ALVAC infected
15   cells.

    Example 27  -  GENERATION OF ALVAC/HIV-1 gag/pol AND
                  env (gp160) RECOMBINANT

        The sequence encoding the 5'-end of the gag
gene was cloned between vaccinia virus tk flanking arms.
20   This was accomplished by initially preparing plasmid
pHIVGE15 as described above (see Example 24).

        The gp120 gene was then replaced by the gp160
gene. This was accomplished by cloning the 2,600 bp
NruI-NotI fragment of pH6HMNE, containing the entire HIV-
25   1(MN) envelope (gp160) gene, into the 9,800 bp NruI-NotI
fragment of pHIVGE15. The plasmid generated by this
manipulation is called pHIVGE18.

        The canary pox flanking arm deleted in the
previous step was then cloned into pHIVGE18. This was
30   accomplished by cloning the 1,500 bp NotI fragment of
pHIVGE15, containing the C3 flanking arm, into the 12,400
bp NotI fragment of pHIVGE18. The plasmid generated by
this manipulation is called pHIVGE20.

        pHIVGE20 was used in recombination experiments
35   with ALVAC (CPpp) as the rescuing virus to yield vCP130.

        Immunoprecipitation analysis was performed with
CEF cell monolayers as described above to determine

whether vCP130 expresses authentic HIV-1 gag and env gene
products.

Lysates from the infected cells were analyzed
for HIV-1 gag and env gene expression using pooled serum
5    from HIV-1 seropositive individuals (obtained from
Chiron, Emeryville, CA). The sera was preadsorbed with
ALVAC infected CEF cells. The preadsorbed sera was bound
to Protein A-sepharose in an overnight incubation at 4°C.
Following this incubation period, the material was washed
10   4X with 1X buffer A. Lysates precleared with normal
human sera and protein A-sepharose were then incubated
overnight at 4°C with the HIV-1 seropositive human sera
bound to protein A-sepharose. After the overnight
incubation period, the samples were washed 4X with 1X
15   buffer A and 2X with a LiCl$_2$/urea buffer. Precipitated
proteins were dissociated from the immune complexes by
the addition of 2X Laemmli's buffer (125 mM Tris (pH6.8),
4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling
for 5 min. Proteins were fractionated on a 10% Dreyfuss
20   gel system (Dreyfuss et al., 1984), fixed and treated
with 1M Na-salicylate for fluorography. Human sera from
HIV-1 seropositive individuals specifically precipitated
the HIV-1 gag and env proteins from vCP130 infected
cells, but did not precipitate HIV-1-specific proteins
25   from mock infected or ALVAC infected cells.

**Example 28    -    GENERATION OF ALVAC/HIV-1 gag/pol
                         RECOMBINANT**

A plasmid, pHXB2D, containing HIV-1 cDNA
sequence was obtained from Dr. R.C. Gallo (NCI-NIH). The
30   sequence encoding the 5'-end of the gag gene was cloned
between vaccinia virus tk flanking arms. This was
accomplished by initially preparing plasmid pHIVG7 as
described above (see Example 16).

The gag and pol genes were then cloned between
35   canary pox flanking arms. This was accomplished by
cloning the 4,400 bp SmaI-NotI fragment of pHIVG7,
containing the I3L-promoted gag and pol genes, and the

oligonucleotides, HIV2L6 (SEQ ID NO:131) (5'-GGCCAAAC-3')
and HIV2L7 (SEQ ID NO:132) (5'-TCGAGTTT-3'), into the
SmaI-XhoI site of pSPCP3L. The plasmid generated by this
manipulation is called pHIVG24.

5              pHIVG24 was used in recombination experiments
with ALVAC (CPpp) as the rescuing virus to yield vCP152.
          Immunoprecipitation experiments with vCP152
infected cells were performed as described above for the
expression of the HIV-1 env and gag proteins. No HIV-1-
10  specific species were precipitated from mock infected or
ALVAC infected cells. Protein species corresponding to
the gag precursor protein, as well as various
intermediate and mature gag cleavage products, were
precipitated, however, from lysates of vCP152 infected
15  cells.

**Example 29  -  GENERATION OF ALVAC/HIV-1 gag/pol AND
              env (TRUNCATED) RECOMBINANT**

          A plasmid, pHXB2D, containing HIV-1 cDNA
sequence was obtained from Dr. R.C. Gallo (NCI-NIH). The
20  sequence encoding the 5'-end of the gag gene was cloned
between vaccinia virus tk flanking arms. This was
accomplished by initially preparing plasmid pHIVG24 as
described above (see Example 28).
          pHIVG24 was used in recombination experiments
25  with vCP120 as the rescuing virus to yield vCP155.
          Immunoprecipitation experiments with vCP155
infected cells were performed as described above for the
expression of the HIV-1 env and gag proteins. No HIV-1-
specific species were precipitated from mock infected
30  cells. Protein species corresponding to the env and gag
precursor proteins, as well as various intermediate and
mature gag cleavage products, were precipitated, however,
from lysates of CP155 infected cells.

96

**Example 30  -  GENERATION OF ALVAC/HIV-1 gag/pol AND env**
**(gp120 WITH TRANSMEMBRANE ANCHOR)**
**RECOMBINANT**

A plasmid, pHXB2D, containing HIV-1 cDNA

5   sequence was obtained from Dr. R.C. Gallo (NCI-NIH).  The
sequence encoding the 5'-end of the gag gene was cloned
between vaccinia virus tk flanking arms.  This was
accomplished by initially preparing plasmid pHIVG24 as
described above (see Example 28).

10   pHIVG24 was used in recombination experiments
with vCP138 as the rescuing virus to yield vCP156.

Immunoprecipitation experiments with vCP156
infected cells were performed as described above for the
expression of the HIV-1 env and gag proteins (CEF Cell

15   monolayer).  No HIV-1-specific species were precipitated
from mock infected cells.  Protein species corresponding
to the env and gag precursor proteins, as well as various
intermediate and mature cleavage products, were
precipitated, however, from lysates of vCP156 infected

20   cells.

Expression of HIV-1 gag-specific gene products
either alone or in combination with env by vaccinia virus
has been shown to lead to the production of non-
infectious virus-like particles (Haffar et al., 1990; Hu

25   et al., 1990).  With this background it was investigated
whether cells infected with ALVAC-based recombinant
expressing HIV-1 gag-pol and env genes would also produce
such particles.  Furthermore, if these ALVAC-based
recombinants were used to infect non-avian cells (i.e.

30   Vero, MRC-5, etc.) then HIV-1 virus-like particles could
be purified without any poxvirus virion contaminants.

To evaluate particle formation using Vero cells
infected with vCP156, the following experiment was
performed.  Vero cells were infected at an m.o.i. of

35   approximately 5 pfu/cell.  After a 24 hr infection
period, the supernatant was harvested and clarified by
centrifugation at 2000 rpm for 10 min.  The supernatant

was then spun through filters which have a molecular
weight cutoff of 30,000 kDa (Centricell 60, Polysciences,
Inc., Warrington, PA). Thus, any smaller molecules would
pass through these filters. The material retained by the
5   filters was then analyzed by standard Western blot
analysis (Maniatis et al., 1990) using pooled human serum
from HIV-seropositive individuals (obtained from Dr. J.
Conroy, New York State Department of Health). The
results from the Western blot analysis demonstrated the
10  presence of the major core protein p24 and the HIV-1(MN)
anchored gp120 in the material retained by the filters.
With the size exclusion noted above, the p24 would have
passed through unless it was in a higher structural
configuration (i.e. virus-like particles). Therefore,
15  these results strongly suggest that HIV-1 virus-like
particles containing the gp120 envelope component are
produced in vCP156 infected cells.

Example 31  -  **EXPRESSION OF THE T1, T2, AND TH4.1**
             **EPITOPES OF THE HIV-1 env GENE IN ALVAC**
20           **AND NYVAC**

Recombinant poxviruses vP1062 and vCP146 were
generated to express the T1, T2, and TH4.1 epitopes of
HIV-1 env (Hosmalin et al., 1991) as individual peptides.

Construction of plasmid p731T1. Plasmid pMPI3H
25  contains the vaccinia I3L early/intermediate promoter
element (Schmitt and Stunnenberg, 1988; Vos and
Stunnenberg, 1988) in a pUC8 background. The promoter
element was synthesized by polymerase chain reaction
(PCR) using pMPVC1, a subclone of vaccinia HindIII I, as
30  template and synthetic oligonucleotides MPSYN283 (SEQ ID
NO:133) (5'- CCCCCCAAGCTTACATCATGCAGTGGTTAAAC -3') and
MPSYN287 (SEQ ID NO:134) (5'- GATTAAACCTAAATAATTGT -3')
as primers. DNA from this reaction was cut with HindIII
and RsaI and a 0.1 kb fragment containing the promoter
35  element was purified. A linker region was assembled by
annealing complementary synthetic oligonucleotides
MPSYN398 (SEQ ID NO:135) (5'- ACAATTATTTAGGTTAACTGCA -3')

and MPSYN399 (SEQ ID NO:136)

(5'- GTTAACCTAAATAATTGT -3').   The PCR-derived promoter
element and the polylinker region were ligated with
vector plasmid pUC8 which had been cut with HindIII and

5   PstI. The resulting plasmid, pMPI3H, contains the I3L
promoter region from positions -100 through -6 relative
to the initiation codon, followed by a polylinker region
containing HpaI, PstI, SalI, BamHI, SmaI and EcoRI sites.
Cleavage with HpaI produces blunt ended DNA linearized at

10  position -6 in the promoter.

        A cassette containing the T1 peptide driven by
the vaccinia I3L promoter was generated by ligating
complementary oligonucleotides T1C (SEQ ID NO:137)
(5'- TAATCATGAAACAAATTATTAATATGTGGCAAGAAGAGGAAAAGCTATGTAC

15  GCTTGACTAGTTAATCACTCGAG -3') and T1N (SEQ ID NO:138)
(5'- GATCCTCGAGTGATTAACTAGTCAAGCGTACATAGCTTTTCCTACTTCTTGC
CACATATTAATAATTTGTTTCATGATTA -3') to plasmid pMPI3H
digested with HpaI and BamHI.  This ligation
reconstitutes the last 5 base pairs of the promoter,

20  provides the complete coding sequence of the T1 peptide,
and creates a XhoI site between the stop codon and BamHI
site.  This is plasmid p731T1.  The sequence of the
fragment was confirmed by nucleotide sequence analysis.

        Construction of plasmid pH6T2.  A cassette

25  containing the T2 peptide driven by the vaccinia H6
promoter was generated in two steps:  The H6 promoter
through the EcoRV site was derived from a plasmid
containing the synthetic H6 promoter (Perkus et al.,
1989), using PCR and primers H6PCR1 (SEQ ID NO:157) and

30  H6PCR2 (SEQ ID NO:205) (5'-TTAACGGATATCGCGATAATG-3')
creating a 5' HindIII site.  This 122 bp PCR-derived
fragment was digested with HindIII and EcoRV followed by
ligation to similarly digested pBS-SK+ (Stratagene, La
Jolla, CA), generating plasmid pBSH6.  Complementary

35  oligonucleotides T2C (SEQ ID NO:139)
(5'- ATCCGTTAAGTTTGTATCGTAATGCACGAAGATATTATTTCTTTGTGGGATC
AATCTTTAAAATGACTAGTTAATCAG -3') and T2N (SEQ ID NO:140)

(5'- GATCCTGATTAACTAGTCATTTTAAAGATTGATCCCACAAAGAAATAATATC
TTCGTGCATTACGATACAAACTTAACGGAT -3') which complete the 3'
end of the H6 promoter from the EcoRV site, encode the T2
peptide and create a BamHI site at the 3' end of the gene

5   were annealed then ligated to pBSH6 that was digested
with EcoRV and BamHI. This plasmid was designated pH6T2
following confirmation of the fragment by nucleotide
sequence analysis.

Construction of plasmid pVQ42KTH4.1. A

10  cassette containing the TH4.1 peptide driven by the AmEPV
42K promoter was generated by sequential PCR reactions:
the 107 bp 42K promoter with 5' PstI and SmaI sites was
derived by PCR from plasmid p42KRABI, a plasmid
containing the gene for the rabies glycoprotein under
15  control of the 42K promoter, using primers 42KVQ1 (SEQ ID
NO:141)

(5'- AATTAATTAGCTGCAGCCCCGGGTCAAAAAAATATAAATG -3') and
42KVQ2 (SEQ ID NO:142)

(5'- CCTTGTACTACTTCAATTACTCTATCCATTTTATATTGTAATTATATATTTT
20  C). The sequence of the 107 bp promoter region of this
PCR-derived fragment is (SEQ ID NO:143)

5'- TCAAAAAAATATAAATGATTCACCATCTGATAGAAAAAAAATTTATTGGGAAG
AATATGATAATATTTTGGGATTTCAAAATTGAAAATATATAATTACAATATAAA
-3. The 159 bp PCR-derived fragment was fused to the
25  coding sequences of TH4.1 with a second PCR using this
fragment and synthetic oligonucleotides encoding the
TH4.1 peptide TH41C (SEQ ID NO:144)

(5'- ATGGATAGAGTAATTGAAGTAGTACAAGGAGCTTATAGAGCTATTAGATGAC
TAGTTAATCACTCGAGGATCC -3') and TH41N (SEQ ID NO:145)

30  (5'- GGATCCTCGAGTGATTAACTAGTCATCTAATAGCTCTATAAGCTCCTTGTAC
TACTTCAATTACTCTATCCAT -3') as template and primers
42KTH41 (SEQ ID NO:146)

(5'- ATCATCGGATCCTCGAGTGATTAAACTAGTCATCTAATAGCTC -3') and
42KVQ1 (SEQ ID NO:141). This 210 bp PCR-derived fragment
35  was extended in the 5' direction, incorporating a BamHI
site at the 5' end using the fragment and synthetic
oligonucleotides VQC (SEQ ID NO:147)

(5'- TTAATCAGGATCCTTAATTAATTAGTTATTAGACAAGGTGAAAACGAAACTA
TTTGTAGCTTAATTAATTAGCTGCAGCCCGGG -3') and VQN (SEQ ID
NO:148)

(5'- CCCGGGCTGCAGCTAATTAATTAAGCTACAAATAGTTTCGTTTTCACCTTGT
5   CTAATAACTAATTAATTAAGGATCCTGATTAA -3') as template for a
third PCR using primers 42KTH41 (SEQ ID NO:146) and BAMVQ
(SEQ ID NO:149)

(5'- TTAATCAGGATCCTTAATTAATTAGTTATTAGAC -3'). Subsequent
nucleotide sequence analysis revealed an error in the
10  sequence of oligonucleotide 42KTH41 (SEQ ID NO:146) such
that an extra base (an A) was inserted after position 24
as indicated by the underline in the above sequence for
42KTH41. This was corrected with a final PCR employing
the 272 bp fragment derived from the third PCR as
15  template with primers BAMVQ (SEQ ID NO:149) and 42KTH41A
(SEQ ID NO:150)

(5'- ATCATCGGATCCTCGAGTGATTAACTAGTCATCTAATAGCTC -3').
After the final PCR, the cassette was to contain BamHI,
PstI, and SmaI sites 5' to 42K-TH4.1 with XhoI and BamHI
20  sites 3'. This 271 bp PCR-derived fragment was digested
with BamHI and cloned into the BamHI site of pBS-SK+
(Stratagene, La Jolla, CA) generating plasmid
pVQ42KTH4.1. Nucleotide sequence analysis of this
plasmid confirmed that the sequence of the promoter and
25  coding region was correct. However, a 3 bp deletion was
revealed resulting in loss of the 3' BamHI site.

        Construction of plasmid pT1T2TH4.1. These
three cassettes were combined into a singular plasmid
pT1T2TH4.1 such that I3L-T1 is opposite in orientation to
30  the other two genes in the following manner: A 170 bp
HindIII/XhoI fragment was isolated from p731T1 and
ligated to similarly digested pH6T2 generating pT1T2. A
290 bp BamHI/SacI fragment from pVQ42KTH4.1 was ligated
to similarly digested pT1T2, creating pT1T2TH4.1. The
35  sequence of the insert was confirmed by nucleotide
sequence analysis.

101

pC5LSP (defined in Example 14) was digested
with EcoRI, treated with alkaline phosphatase and ligated
to self-annealed oligonucleotide CP29 (SEQ ID NO:151)
(5'-AATTGCGGCCGC-3'), digested with NotI and linear
5  purified followed by self-ligation.  This procedure
introduced a NotI site to pC5LSP, generating pNC5LSP5.
       Plasmid pSD550 was derived from pSD548 as
follows.  Plasmid pSD548 (Tartaglia et al., 1992) is a
vaccinia vector plasmid in which the I4L ORF (Goebel et
10  al., 1990a,b) is replaced by a cloning region consisting
of BglII and SmaI sites.  To expand the multicloning
region, pSD548 was cut with BglII and SmaI and ligated
with annealed complementary synthetic oligonucleotides
539A (SEQ ID NO:152) (5'-
15  AGAAAAATCAGTTAGCTAAGATCTCCCGGGCTCGAGGGTACCGGATCCTGATTAGTT
AATTTTTGT-3') AND 539B (SEQ ID NO:153) (5'-
GATCACAAAAATTAACTAATCAGGATCCGGTACCCTCGAGCCCGGGAGATCTTAGCT
AACTGATTTTTCT-3').  In the resulting plasmid, pSD550, the
multicloning region contains BglII, SmaI, XhoI, KpnI and
20  BamHI restriction sites.
       The 602 bp XhoI fragment from pT1T2TH4.1
containing the genes for the epitopes driven by their
respective promoters was cloned into donor plasmids
pNC5LSP5 and pSD550 in their XhoI sites.  Nucleotide
25  sequence analysis was used to confirm the sequence and
the orientation of the insert.  The resulting plasmids
pC5T1T2TH4.1 and pI4T1T2TH4.1 were used in in vitro
recombination experiments with ALVAC and NYVAC to
generate recombinant viruses vCP146 and vP1062,
30  respectively.  These recombinant viruses were
demonstrated to contain the desired genes by
hybridization of a specific DNA probe to viral plaques.

Example 32 .- EXPRESSION OF TWO FUSION PEPTIDES
CONTAINING THE T1, T2, AND TH4.1 EPITOPES
OF HIV-1 env WITH AND WITHOUT A
TRANSMEMBRANE ANCHOR DOMAIN FROM HIV-1 env

Recombinant poxviruses vP1060, vP1061, vCP154
and vCP148 were created to express a fusion peptide
consisting of the signal sequences from HIV-1 env coupled
to sequences corresponding to the T1, T2, and TH4.1
epitopes of HIV-1 env by cleavable linker regions.
vP1060 and vCP154 differ from vP1061 and vCP148 in that
the former recombinant viruses express the fusion peptide
along with sequences corresponding to the transmembrane
region of HIV-1 env.

Both fusion peptides include the 51 amino acid
N-terminal portion of HIV-1 (IIIB) env, residues 1-50
(plus initiating Met) based on Ratner et al. (1985). The
amino acid sequence of this signal region (SEQ ID NO:154)
is MKEQKTVAMRVKEKYQHLWRWGWRWGTMLLGMLMICSATEKLWVTVYYGVP.
This is followed by the T1, T2, and TH4.1 epitopes
(Hosmalin et al., 1991) separated from the signal, each
other, and anchor sequence where present, by a cleavable
linker region up to 5 amino acids in length. The amino
acid sequence of this region of the peptide (SEQ ID
NO:155) is [signal]-PFR-KQIINMWQEVGKAMYA-PPFRK-
HEDIISLWDQSLK-PPFRK-DRVIEVVQGAYRAIR-[PPFRK-anchor]. The
anchor domain is a 28 amino acid transmembrane region of
HIV-1 (IIIB) env, residues 691-718. The amino acid
sequence of this region (SEQ ID NO:156) is
LFIMIVGGLVGLRIVFAVLSVVNRVRQG-[stop].

For both versions of the fusion peptide, the H6
promoter and HIV-1 env signal sequences were derived by
PCR from plasmid pBSH6HIV3B5P (defined in Example 2)
using primers H6PCR1 (SEQ ID NO:157)
(5'ACTACTAAGCTTCTTTATTCTATACTTAAAAAGTG-3') and PCRSIGT1
(SEQ ID NO:158)
(5'- CATATTAATTTGTTTTCTAAAAGGAGGTACCCCATAATAGACTGTG -3').
This 314 bp PCR-derived fragment consists of a 5' HindIII

site followed by the H6 promoter and coding sequences for
the signal, linker, and the first 6 amino acids of the T1
peptide.

The remainder of the coding region for the
5   construct without the transmembrane region was generated
by PCR amplification of oligonucleotides T2T4A (SEQ ID
NO:159)
(5'- GCTCCTCCTTTTAGAAAACACGAAGATATTATTTCTTTGTGGGATCAATCTT
TAAAACCTCCTTTTAGAAAAGATAGAGTAATTGAAGTAGTAC -3') and T2T4B
10  (SEQ ID NO:160)
(5'- GTACTACTTCAATTACTCTATCTTTTCTAAAAGGAGGTTTTAAAGATTGATC
CCACAAAGAAATAATATCTTCGTGTTTTCTAAAAGGAGGAGC -3') using
primers PCRT1T2 (SEQ ID NO:161)
(5'- AAACAAATTATTAATATGTGGCAAGAAGTAGGAAAAGCTATGTACGCTCCTC
15  CTTTTAGAAAACACGAAG -3') and PCRT4END (SEQ ID NO:162)
(5'- ACTACTTCTAGATTATCTAATAGCTCTATAAGCTCCTTGTACTACTTCAATT
ACTC -3'). This 177 bp PCR-derived fragment encodes the
T1 peptide, a linker region, the T2 peptide, another
linker region, and the TH4.1 peptide, followed by a 3'
20  XbaI site. This fragment was fused with the 314 bp PCR-
derived fragment containing the promoter and signal
sequences by PCR with primers H6PCR1 (SEQ ID NO:157) and
PCRT4END (SEQ ID NO:162). Following digestion of this
473 bp PCR-derived fragment with HindIII and XbaI a
25  fragment of 455 bp was isolated from an agarose gel,
ligated to similarly digested pBS-SK, and the sequence of
the insert verified by nucleotide sequence analysis. The
resulting plasmid was designated pBST1T2TH4.1.

The remainder of the coding region of the
30  version with the transmembrane anchor was generated by
PCR amplification of oligonucleotides, T2T4A (SEQ ID
NO:159) and T2T4B (SEQ ID NO:160), using primers PCRT1T2
(SEQ ID NO:161) and PCRT4TM (SEQ ID NO:163)
(5'- TACTATCATTATGAATAATTTTCTAAAAGGAGGTCTAATAGCTCTATAAGCT
35  CCTTGTACTACTTCAATTACTC -3'), altering the 3' end to
accommodate the transmembrane region. This 195 bp PCR-
derived fragment was fused by PCR with oligonucleotides

comprising the anchor, HIVTM1 (SEQ ID NO:107) and HIVTM2
(SEQ ID NO:108) using primers PCRT1T2 (SEQ ID NO:161) and
PCRT4END (SEQ ID NO:162). This 276 bp PCR-derived
fragment was fused with the 314 bp PCR-derived fragment

5   containing the promoter and signal sequences by PCR with
primers H6PCR1 (SEQ ID NO:157) and PCRT4END (SEQ ID
NO:162). Following digestion of this 572 bp PCR-derived
fragment with HindIII and XbaI a fragment of 554 bp was
isolated from an agarose gel, ligated to similarly

10  digested pBS, and the sequence of the insert verified by
nucleotide sequence analysis. The resulting plasmid was
designated pBST1T2TH4.1A.

         pC5LSP (defined in Example 14) was digested
with BamHI and ligated to annealed oligonucleotides CP32

15  (SEQ ID NO:112) and CP33 (SEQ ID NO:113) to generate
pVQC5LSP6. pVQC5LSP6 was digested with EcoRI, treated
with alkaline phosphatase and ligated to self-annealed
oligonucleotide CP29 (SEQ ID NO:151), digested with NotI
and linear purified followed by self-ligation. This

20  procedure introduced a NotI site to pVQC5LSP6, generating
pNVQC5LSP7.

         Both cassettes were placed individually between
the XhoI and XbaI sites of insertion plasmid pNVQC5LSP7.
These plasmids pC5ST1T1TH4.1 and pC5ST1T2TH4.1A were used

25  to generate canarypoxvirus recombinants, vCP148 and
vCP154 respectively. BamHI-SmaI fragments were excised
from pC5ST1T1TH4.1 and pC5ST1T2TH4.1A and ligated to
similarly digested pSD550 (defined in Example 31)
generating plasmids pI4ST1T2TH4.1 and pI4ST1T1TH4.1A,

30  respectively. These plasmids were used in recombination
experiments with NYVAC as the rescuing virus resulting in
recombinants vP1061 and vP1060, respectively. These
recombinant viruses were demonstrated to contain the
desired genes by hybridization of a specific DNA probe to

35  viral plaques.

**Example 33  -  EXPRESSION OF THE HIV-1 *nef* GENE IN ALVAC,**
**TROVAC, AND NYVAC**

Recombinant poxviruses vP1084, vFP174, and
vCP168 were generated expressing HIV-1 *nef* (MN) as
5   follows:

The I3L promoter was derived by PCR from
plasmid pMPI3H using primers I3PCR1 (SEQ ID NO:164)
(5'- ATCATCGGATCCAAGCTTACATCATGCAGTGG -3') and PI3NEF2
(SEQ ID NO:165)
10  (5'- CGTTTTGACCATTTGCCACCCATGATTAAACCTAAATAATTGTACTTTG -3
'). The coding region of *nef* was generated by PCR
amplification of pMN1.8-10 (a clone of that portion of
the MN genome between <u>Sst</u>I sites at 7792 and 9595) using
primers PI3NEF1 (SEQ ID NO:166)
15  (5'- AGTACAATTATTTAGGTTTAATCATGGGTGGCAAATGGTCAAAACG -3')
and PNEFBAM (SEQ ID NO:167)
(5'- ATCATCGGATCCTAACACTTCTCTCTCCGG -3'). Fusion of the
coding region with the promoter was accomplished by
amplification of the previous PCR products using primers
20  I3PCR1 (SEQ ID NO:164) and PNEFBAM (SEQ ID NO:167).
Following digestion of this product with <u>Bam</u>HI a fragment
of 0.7 kb was isolated from an agarose gel and ligated to
similarly digested pBS-SK+ (Stratagene, La Jolla, CA),
generating plasmid pI3NEF. It was at this point that
25  sequence deviations were first observed. The sequence of
the cassette was determined to differ from the published
sequence (Gurgo et al., 1988, GenBank Accession Number
M17449) due to deviations in plasmid pMN1.8-10 (provided
by Dr. R.C. Gallo, NCI-NIH). These differences are
30  summarized below relative to the published sequence.

| Base # | Gurgo et al. | pMN1.8-10 | Result | Cassette |
|--------|--------------|-----------|--------|----------|
| 8834 | T | T | silent | C (aa=Arg) |
| 8863 | G | A | Lys->Arg | A (aa=Lys) |
| 8890 | T | C | Pro->Leu | C (aa=Pro) |
| 35   9028 | A | G | Arg->Lys | G (aa=Arg) |
| 9127 | A | A | silent | G (aa=Gln) |
| 9330-9331 | GG | GGG | frameshift | GG |

The two silent mutations in the cassette (at positions
8834 and 9127). were apparently errors in PCR. Since
there is no effect on the encoded protein, these were
allowed to persist. The frameshift at 9930 results in a
5   lengthened open reading frame more closely resembling
other HIV-1 isolates. In keeping with the published size
of *nef* from the MN isolate, this cassette required a
fourth PCR to generate the truncated 3' end of the coding
region.

10          Removal of the extra base at position 9930 was
accomplished by PCR amplification of the insert in pI3NEF
with primers I3PCR1 (SEQ ID NO:164) and PNEFFIX1 (SEQ ID
NO:168)
(5'- ATCATCGGATCCTAACACTTCTCTCTCCGGGTCATCCATCCATGCTGGCTCA
15   TAG -3'). Following digestion of this 678 bp PCR-derived
fragment with BamHI a fragment of 660 bp was isolated
from an agarose gel and ligated to similarly digested
pBS, generating plasmid pBSI3NEF. The insert was
verified by nucleotide sequence analysis.

20          The 660 bp BamHI fragment from pBSI3NEF
containing the *nef* gene was placed in the BamHI site of
insertion plasmid pNVQC5LSP7 (defined in Example 32).
The resulting plasmid pC5I3NEF was employed in a
recombination into the C5 locus of ALVAC, generating the
25   recombinant vCP168. The same 660 bp BamHI fragment was
also placed in the BamHI site of insertion plasmid pSD550
(defined in Example 31), creating plasmid pI4I3NEF. A
recombination with this plasmid with NYVAC generated
recombinant vP1084.

30          An insertion plasmid for the F16 locos of
TROVAC was derived in the following manner. A 7.3 kb
NaeI/NdeI fragment was isolated from a plasmid containing
the 10.5 kb HindIII fragment of fowlpox virus described
by Tartaglia et al. (1990) and ligated to similarly
35   digested pUC9 creating plasmid pRW866.

          pUC9 was digested with PvuII and an EcoRI
linker ligated between the PvuII sites creating plasmid

pRW715.  A cloning site flanked by fowlpox sequences was
generated by PCR amplification of a portion of the 10.5
kb fragment with primers RW264 (SEQ ID NO:169)
(5'- AATTAACCCGGGATCCAAGCTTCTAGCTAGCTAATTTTTATAGCGGCCGCTA
5  TAATCGTTAACTTATTAG -3') and RW267 (SEQ ID NO:170)
(5'- GCTAGAAATCTCTTAGTTTTTATAGTTG -3').  An adjacent
region was also amplified by PCR using primers RW266 (SEQ
ID NO:171) (5'- GTTACATATGTACAGAATCTGATCATAG -3') and
RW265 (SEQ ID NO:172)
10  (5'- CTAGCTAGAAGCTTGGATCCCGGGTTAATTAATTAATAAAAAGCGGCCGCGT
TAAAGTAGAAAAATG -3').  These PCR-derived fragments were
fused by a third PCR using primers RW266 (SEQ ID NO:171)
and RW267 (SEQ ID NO:170).  The resulting PCR-derived
fragment consisted of fowlpox sequences flanked by a 5'
15  EcoRI site and a 3' NdeI site.  Central in the fragment
is a polycloning region containing SmaI, BamHI, and
HindIII sites, flanked by NotI sites and translation stop
codons in six reading frames.  An early transcription
termination signal (Yuen and Moss, 1987) is adjacent to
20  the 3' NotI site.  This PCR-derived fragment, digested
with EcoRI and NdeI, was ligated to similarly digested
pRW715 creating plasmid pRW864.  An 11K promoted lac Z
gene was excised from pAM1 by partial BamHI, total PstI
digestion.  This fragment was made blunt ended with
25  Klenow polymerase and ligated to SmaI digested pRW864,
creating pRW867A.  The NotI fragment from pRW867A was
made blunt ended with Klenow polymerase in the presence
of dNTPs so that the NotI sites would be regenerated when
ligated into an FspI site, and ligated to pRW866 which
30  was partially digested with FspI such that the insertion
was made corresponding to position 1955 described by
Tartaglia et al. (1990).  The resulting plasmid, pRW868,
was then digested with NotI to remove the lac Z cassette,
and ligated to the 66 bp polylinker from pRW864 which was
35  excised by NotI digestion.  The resulting plasmid was
designated pRW673.  An 81 bp SmaI fragment was derived

from pVQ42KTH4.1 (defined in Example 31) and inserted
into SmaI digested pRW873 generating plasmid pVQ873.

      The *nef* cassette was excised from pBSI3NEF as a
684 bp HindIII fragment for insertion into pVQ873

5 followed by recombination into the F16 locus of TROVAC to
generate vFP174.

**Example 34**   -  **EXPRESSION OF HIV-2 GENES IN NYVAC**

      Generation of NYVAC/HIV2 gag/pol recombinant.

A plasmid, pISSYEGP, containing the human

10 immunodeficiency virus type 2 (HIV2) gag and pol genes
was obtained from Dr. G. Franchini (NCI-NIH).  The gag
and pol genes from this plasmid were cloned downstream of
the I3L promoter and between vaccinia virus tk flanking
arms.  This was accomplished by cloning the 4,400 bp

15 BstUI-BglII fragment of pISSYEGP, containing the HIV2 gag
and pol genes, and the oligonucleotides, SIVL1 (SEQ ID
NO:69) and HIV2L1 (SEQ ID NO:173) (5'-
CGCCCATGATTAAACCTAAATAATTGTACTTTGTAATATAATGATATATATTTTCAC
TTTATCTCAC-3'), containing the I3L promoter, into the

20 4,070 bp XhoI-BglII fragment of pSD542 (defined in
Example 15).  The plasmid generated by this manipulation
is called pHIV21.

      Extraneous 3'-noncoding sequence was then
eliminated.  This was accomplished by cloning a 280 bp

25 BclI-SmaI PCR fragment, containing the 3'-end of the pol
gene, into the 8,100 bp BclI-SmaI fragment of pHIV21.
This PCR fragment was generated from the plasmid,
pISSYEGP, with the oligonucleotides, HIV2P2 (SEQ ID
NO:174) (5'-ATGGCAGTTCATTGCAT-3') and HIV2P3 (SEQ ID

30 NO:175) (5'-TTCCCGGGAGATCTCTATGCCATTTCTCCAT-3').  The
plasmid generated by this manipulation is called pHIV22.

      pHIV22 was used in recombination experiments
with NYVAC (vP866) as the rescuing virus to yield vP1045.

      Immunoprecipitation analysis was performed to

35 determine whether vP1045 expresses authentic HIV2 gag
gene products.

109

Lysates from the infected cells were analyzed
for HIV2 gag expression using pooled serum from HIV2
seropositive individuals (obtained from Dr. G. Franchini
(NCI-NIH)). The sera was preadsorbed with vP866 infected
5  Vero cells. The preadsorbed sera was bound to Protein A-
sepharose in an overnight incubation at 4°C. Following
this incubation period, the material was washed 4X with
1X buffer A. Lysates precleared with normal human sera
and protein A-sepharose were then incubated overnight at
10  4°C with the HIV2 seropositive human sera bound to
protein A-sepharose. After the overnight incubation
period, the samples were washed 4X with 1X buffer A and
2X with a LiCl$_2$/urea buffer. Precipitated proteins were
dissociated from the immune complexes by the addition of
15  2X Laemmli's buffer (125 mM Tris (pH6.8), 4% SDS, 20%
glycerol, 10% 2-mercaptoethanol) and boiling for 5 min.
Proteins were fractionated on a 10% Dreyfuss gel system
(Dreyfuss et al., 1984), fixed and treated with 1M Na-
salicylate for fluorography.

20            Human sera from HIV2 seropositive individuals
specifically precipitated the HIV2 gag precursor protein,
as well as various intermediate and mature gag cleavage
protein products, from vP1045 infected cells, but did not
precipitate HIV2-specific proteins from mock infected or
25  NYVAC infected cells.

Example 35   -   GENERATION OF NYVAC/HIV2 gag/pol AND
                 env (gp160) RECOMBINANT

A plasmid, pISSYEGP, containing the HIV2 gag
and pol genes was obtained from Dr. G. Franchini (NCI-
30  NIH). The gag and pol genes from this plasmid were
cloned downstream of the I3L promoter and between
vaccinia virus tk flanking arms. This was accomplished
by initially preparing plasmid pHIV22 as described above
(see Example 34).

35            pHIV22 was used in recombination experiments
with vP920 as the rescuing virus to yield vP1047.

Immunoprecipitation experiments with vP1047
infected cells were performed as described above for the
expression of the HIV2 gag proteins.  No HIV2-specific
species were precipitated from mock infected cells.

5    Protein species corresponding to the HIV2 env and gag
precursor proteins, as well as various intermediate and
mature gag cleavage products, were precipitated, however,
from lysates of vP1047 infected cells.

Example 36   -  GENERATION OF NYVAC/HIV2 gag/pol AND

10                   env (gp120) RECOMBINANT

A plasmid, pISSYEGP, containing the HIV2 gag
and pol genes was obtained from Dr. G. Franchini (NCI-
NIH).  The gag and pol genes from this plasmid were
cloned downstream of the I3L promoter and between

15   vaccinia virus tk flanking arms.  This was accomplished
by initially preparing plasmid pHIV22 as described above
(see Example 34).

pHIV22 was used in recombination experiments
with vP922 as the rescuing virus to yield vP1044.

20          Immunoprecipitation experiments with vP1044
infected cells were performed as described above for the
expression of the HIV2 gag proteins.  No HIV2-specific
species were precipitated from mock infected cells.
Protein species corresponding to the HIV2 env and gag

25   precursor proteins, as well as various intermediate and
mature gag cleavage products, were precipitated, however,
from lysates of vP1044 infected cells.

Example 37   -  EXPRESSION OF HIV2 GENES IN ALVAC

Generation of ALVAC/HIV2 gag/pol and env

30   (gp160) recombinant.  The plasmid, pBSH6HIV2ENV (defined
in Example 4), contains the H6-promoted HIV2 env (gp160)
gene.  The H6-promoted env gene from this plasmid was
cloned between canary pox flanking arms.  This was
accomplished by cloning the 2,700 bp XhoI-SacII fragment

35   of pBSH6HIV2ENV, containing the H6-promoted env gene, and
the oligonucleotides, HIV2L4 (SEQ ID NO:176) (5'-GGTTG-
3') and HIV2L5 (SEQ ID NO:177) (5'-AATTCAACCGC-3'), into

111

the XhoI-EcoRI site of pC6L (defined in Example 50). The
plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned
into pHIV23. This was accomplished by cloning the 4,450
5  bp XmaI-NotI fragment of pHIV22, containing the I3L-
promoted HIV2 gag and pol genes, and the
oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ
ID NO:132), into the 7,000 bp XmaI-XhoI fragment of
pHIV23. The plasmid generated by this manipulation is
10  called pHIV25.

pHIV25 was used in recombination experiments
with ALVAC (CPpp) as the rescuing virus to yield vCP153.

Immunoprecipitation analysis was performed as
described above, but with CEF cell monolayers to
15  determine whether vCP153 expresses authentic HIV2 gag and
env gene products.

Lysates from the infected cells were analyzed
for HIV2 gag and env gene expression using pooled serum
from HIV2 seropositive individuals (obtained from Dr. G.
20  Franchini (NCI-NIH)). The sera was preadsorbed with
ALVAC infected CEF cells. The preadsorbed sera was bound
to Protein A-sepharose in an overnight incubation at 4°C.
Following this incubation period, the material was washed
4X with 1X buffer A. Lysates precleared with normal
25  human sera and protein A-sepharose were then incubated
overnight at 4°C with the HIV2 seropositive human sera
bound to protein A-sepharose. After the overnight
incubation period, the samples were washed 4X with 1X
buffer A and 2X with a LiCl$_2$/urea buffer. Precipitated
30  proteins were dissociated from the immune complexes by
the addition of 2X Laemmli's buffer (125 mM Tris (pH6.8),
4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling
for 5 min. Proteins were fractionated on a 10% Dreyfuss
gel system (Dreyfuss et al., 1984), fixed and treated
35  with 1M Na-salicylate for fluorography.

Human sera from HIV2 seropositive individuals
specifically precipitated the HIV2 env and gag precursor

proteins, as well as various intermediate and mature gag
cleavage products, from vCP153 infected cells, but did
not precipitate HIV2-specific proteins from mock infected
or ALVAC infected cells.

5   **Example 38   -   EXPRESSION OF SIV GENES IN NYVAC**
                       **GENERATION OF NYVAC/SIV env (gp120-gp28)**
                       **AND gag (PROTEASE⁻) RECOMBINANT**

        Immunoprecipitation analysis was performed to
    determine whether vP948 (defined in Example 5) expresses
10  authentic SIV env and gag precursor proteins.

        Lysates from the infected cells were analyzed
    for SIV env and gag precursor expression using serum from
    SIV seropositive macaques (obtained from Dr. G. Franchini
    (NCI-NIH)).  The sera was preadsorbed with NYVAC (vP866)
15  infected Vero cells.  The preadsorbed sera was bound to
    Protein A-sepharose in an overnight incubation at 4°C.
    Following this incubation period, the material was washed
    4X with 1X buffer A.  Lysates precleared with normal
    macaque sera and protein A-sepharose were then incubated
20  overnight at 4°C with the SIV seropositive macaque sera
    bound to protein A-sepharose.  After the overnight
    incubation period, the samples were washed 4X with 1X
    buffer A and 2X with a LiCl$_2$/urea buffer.  Precipitated
    proteins were dissociated from the immune complexes by
25  the addition of 2X Laemmli's buffer (125 mM Tris (pH6.8),
    4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and boiling
    for 5 min.  Proteins were fractionated on a 10% Dreyfuss
    gel system (Dreyfuss et al., 1984), fixed and treated
    with 1M Na-salicylate for fluorography.

30          Macaque sera from SIV seropositive individuals
    specifically precipitated the SIV gag precursor protein
    and the envelope glycoprotein from vP948 infected cells,
    but did not precipitate SIV-specific proteins from mock
    infected cells.

35  **Example 39   -   GENERATION OF NYVAC/SIV gag/pol**
                       **RECOMBINANT**

A plasmid, pSIVGAGSS11G, containing SIV$_{MAC142}$
cDNA sequence was obtained from Dr. G. Franchini (NCI-
NIH). The gag and pol genes from this plasmid were
cloned downstream of the I3L promoter and between
5  vaccinia virus tk flanking arms. This was accomplished
by preparing plasmid pSIVG5 as described above (see
Example 5).

pSIVG5 was used in recombination experiments
with NYVAC (vP866) as the rescuing virus to yield vP1042.
10  Immunoprecipitation experiments with vP1042
infected cells were performed as described above for the
expression of the SIV env and gag precursor proteins. No
SIV-specific species were precipitated from mock infected
or NYVAC infected Vero cells. Protein species
15  corresponding to the gag precursor protein, as well as
various intermediate and mature gag cleavage products,
were precipitated, however, from lysates of vP1042
infected cells.

**Example 40  -  GENERATION OF NYVAC/SIV gag/pol AND**
20  **env (gp120-gp41) RECOMBINANT**

pSIVG5 (Example 5) was used in recombination
experiments with vP1050 as the rescuing virus to yield
vP1071.

Immunoprecipitation experiments with vP1071
25  infected cells show expression of SIV genes.

**Example 41  -  GENERATION OF NYVAC/SIV gag/pol AND**
**env (gp120-gp28) RECOMBINANT**

pSIVG5 (Example 5) was used in recombination
experiments with vP874 as the rescuing virus to yield
30  vP943.

Immunoprecipitation experiments with vP943
infected cells were performed as described above for the
expression of the SIV env and gag precursor proteins. No
SIV-specific species were precipitated from mock infected
35  Vero cells. Protein species corresponding to the env and
gag precursor proteins, as well as various intermediate

114

and mature gag cleavage products, were precipitated,
however, from lysates of vP943 infected cells.

Example 42 - GENERATION OF NYVAC/SIV p16, p28
          RECOMBINANT

5        Immunoprecipitation experiments with vP942
(Example 5) infected cells were performed as described
above for the expression of the SIV env and gag precursor
proteins. No SIV-specific species were precipitated from
mock infected Vero cells. Protein species corresponding
10 to p16 and p28 were precipitated, however, from lysates
of vP942 infected cells.

Example 43 - GENERATION OF NYVAC/SIV p16, p28 AND
          env (gp120-gp28) RECOMBINANT

         Immunoprecipitation experiments with vP952
15 (Example 5) infected cells were performed as described
above for the expression of the SIV env and gag precursor
proteins. No SIV-specific species were precipitated from
mock infected Vero cells. Protein species corresponding
to env and p16 and p28 were precipitated, however, from
20 lysates of vP952 infected cells.

Example 44 - GENERATION OF NYVAC/SIV env (gp120-gp41)
          RECOMBINANT

         The plasmid, pSIVEMVC, contains the H6-promoted
SIV$_{MAC142}$ envelope gene (in vitro selected truncated
25 version). The region of the envelope gene containing the
premature termination codon was cloned into pBSK+. This
was accomplished by cloning the 1,120 bp ClaI-BamHI
fragment of pSIVEMVC into the ClaI-BamHI site of pBSK+.
The plasmid generated by this manipulation is called
30 pSIV10.

         The upstream termination codon, TAG, was then
changed to the original CAG codon. This was accomplished
by cloning the oligonucleotides, SIVL20 (SEQ ID NO:178)
(5'-
35 CTAGCTAAGTTAAGGCAGGGGTATAGGCCAGTGTTCTCTTCCCCACCCTCTTATTTC
CAGCAGACTCATACCCAACAG-3') and SIVL21 (SEQ ID NO:179) (5'-
GTCCTGTTGGGTATGAGTCTGCTGGAAATAAGAGGGTGGGGAAGAGAACACTGGCCT

ATACCCCTGCCTTAACTTAG-3'), into the 4,000 bp NheI-PpuMI
fragment of pSIV10. The plasmid generated by this
manipulation is called pSIV11.

   The region containing the modified codon was
5  then cloned back into pSIVEMVC. This was accomplished by
cloning the 380 bp BglII-NheI fragment of pSIV11,
containing the modified codon, into the 5,600 bp partial
BglII-NheI fragment of pSIVEMVC. The plasmid generated
by this manipulation is called pSIV12.

10      pSIV12 was used in in vitro recombination
experiments with NYVAC (vP866) as the rescuing virus to
yield vP1050.

   Immunoprecipitation experiments with vP1050
infected cells were performed as described above for the
15 expression of the SIV env and gag precursor proteins. No
SIV-specific species were precipitated from mock infected
or NYVAC infected Vero cells. A protein species
corresponding to env was precipitated, however, from
lysates of vP1050 infected cells.

20 **Example 45   -   EXPRESSION OF SIV GENES IN ALVAC**
   Generation of ALVAC/SIV gag/pol recombinant. A
plasmid, pSIVGAGSS11G, containing SIV$_{MAC142}$ cDNA sequence
was obtained from Dr. G. Franchini (NCI-NIH). The gag
and pol genes from this plasmid were cloned downstream of
25 the I3L promoter and between vaccinia virus flanking
arms. This was accomplished by initially preparing
plasmid pSIVG5 as described above (see Example 5).

   The gag/pol genes were then cloned between
canary pox flanking arms. This was accomplished by
30 cloning the 4,500 bp SmaI-NotI fragment of pSIVG5,
containing the I3L-promoted gag/pol genes, into the SmaI-
NotI site of pC5L (defined in Example 10). The plasmid
generated by this manipulation is called pSIVGC13.

   pSIVGC13 was used in recombination experiments
35 with ALVAC (CPpp) as the rescuing virus to yield vCP172.

   Immunoprecipitation experiments with vCP172
infected cells show expression of SIV genes.

**Example 46 - EXPRESSION OF SIV GENES IN ALVAC**

Generation of ALVAC/SIV env (gp120-gp41) and
gag/pol Recombinant. A plasmid, pSIVGAGSS11G, containing
simian immunodeficiency virus (SIV$_{mac142}$) cDNA sequence

5   was obtained from Genoveffa Franchini (NCI-NIH). The
gag/pol genes from this plasmid were cloned downstream of
the I3L promoter and between vaccinia virus flanking
arms. This was accomplished by cloning the 4,800 bp
CfoI-TaqI fragment of pSIVGAGSS11G, containing the

10  gag/pol genes, and the oligonucleotides, SIVL1 (SEQ ID
NO:69) and SIVL2 (SEQ ID NO:70), encoding the vaccinia
virus I3L promoter, into the 4,070 bp XhoI-AccI fragment
of pSD542VCVQ. The plasmid generated by this
manipulation is called pSIVG1.

15          Extraneous 3'-noncoding sequence was then
eliminated. This was accomplished by cloning a 1,000 bp
BamHI-HpaI PCR fragment, containing the 3'-end of the pol
gene, into the 7,400 bp partial BamHI-HpaI fragment of
pSIVG1. (This PCR fragment was generated from the

20  plasmid, pSIVGAGSS11G, with the oligonucleotides, SIVP5
(SEQ ID NO:71) and SIVP6 (SEQ ID NO:72)). The plasmid
generated by this manipulation is called pSIVG4.

Sequencing analysis revealed that pSIVG4
contains a single base pair deletion within the pol gene.

25  To correct this error the 2,320 bp BglII-StuI fragment of
pSIVG1 was cloned into the 6,100 bp partial BglII-StuI
fragment of pSIVG4. The plasmid generated by this
manipulation is called pSIVG5.

The gag/pol genes were then cloned between

30  canary pox flanking arms. This was accomplished by
cloning the 4,500 bp SmaI-NotI fragment of pSIVG5,
containing the I3L-promoted gag/pol genes, into the SmaI-
NotI site of pC5L. The plasmid generated by this
manipulation is called pSIVGC13.

35          The SIV env gene was then cloned into pSIVGC13.
This was accomplished by cloning the E. coli DNA
polymerase I (Klenow fragment) filled-in 2,750 bp partial

BglII-XhoI fragment of pSIV12, containing the H6-promoted
SIV env gene, into the SmaI site of pSIVGC13. The
plasmid generated by this manipulation is called
pSIVGC14.

5          pSIVGC14 is used in *in vitro* recombination
experiments with ALVAC as the rescuing virus.

Generation of ALVAC/SIV env (gp120-gp41)
Recombinant. A plasmid, pSIVEMVC, contains the H6-
promoted simian immunodeficiency virus envelope gene *(in
10  vitro* selected truncated version). The region of the
envelope gene containing the premature termination codon
was cloned into pBSK+. This was accomplished by cloning
the 1,120 bp ClaI-BamHI fragment of pSIVEMVC into the
ClaI-BamHI site of pBSK+. The plasmid generated by this
15  manipulation is called pSIV10.

The upstream termination codon, TAG, was then
changed to the original CAG codon. This was accomplished
by cloning the oligonucleotides, SIVL20 (SEQ ID NO:178)
and SIVL21 (SEQ ID NO:179), into the 4,000 bp NheI-PpuMI
20  fragment of pSIV10. The plasmid generated by this
manipulation is called pSIV11.

The region containing the modified codon was
then cloned back into pSIVEMVC. This was accomplished by
cloning the 380 bp BglII-NheI fragment of pSIV11 into the
25  5,600 bp partial BglII-NheI fragment of pSIVEMVC. The
plasmid generated by this manipulation is called pSIV12.

The env gene was then cloned between canary pox
flanking arms. This was accomplished by cloning the
2,700 bp NruI-Asp718 fragment of pSIV12, containing the
30  H6-promoted env gene, into the 7,400 bp NruI-Asp718
fragment of pNVQH6C5SLP18. The plasmid generated by this
manipulation is called pSIVGC15.

pSIVGC15 is used in *in vitro* recombination
experiments with ALVAC as the rescuing virus.
35  **Example 47  -  GENERATION OF HIV1 GENES IN ALVAC**
Generation of ALVAC/HIV1 gag (+pro) (IIIB) and
gp120 (+ transmembrane) (MN) Recombinant. A plasmid,

pHXB2D, containing human immunodeficiency virus type 1
(HIV1) cDNA sequence (IIIB) was obtained from Dr. R.C.
Gallo (NCI-NIH). The sequence encoding the 5'-end of the
gag gene was cloned between vaccinia virus tk flanking
5   arms. This was accomplished by cloning the 1,625 bp
BglII fragment of pHXB2D, containing the 5'-end of the
gag gene, into the 4,075 bp BglII fragment of pSD542VCVQ.
The plasmid generated by this manipulation is called
pHIVG2.

10          The 3'-end of the gag gene was then cloned into
pHIVG2. This was accomplished by cloning a 280 bp ApaI-
BamHI PCR fragment, containing the 3'-end of the gag
gene, into the 5,620 bp ApaI-BamHI fragment of pHIVG2.
(This PCR fragment was generated from the plasmid,
15  pHXB2D, with the oligonucleotides, HIVP5 (SEQ ID NO:116)
and HIVP6 (SEQ ID NO:117)). The plasmid generated by
this manipulation is called pHIVG3.
           The I3L promoter was then cloned upstream of
the gag gene. This was accomplished by cloning the
20  oligonucleotides, HIVL17 (SEQ ID NO:118) and HIVL18 (SEQ
ID NO:119), encoding the vaccinia virus I3L promoter and
the 5'-end of the gag gene, into the 5,540 bp partial
BglII-ClaI fragment of pHIVG3. The plasmid generated by
this manipulation is called pHIVG4.

25          The portion of the gag gene encoding p24, p2,
p7 and p6 was then eliminated. This was accomplished by
cloning the oligonucleotides, HIVL19 (SEQ ID NO:120) and
HIVL20 (SEQ ID NO:121), into the 4,450 bp partial PvuII-
BamHI fragment of pHIVG4. The plasmid generated by this
30  manipulation is called pHIVG5.
           The remainder of the gag gene, as well as the
pol gene, was then cloned downstream of the p17 "gene".
This was accomplished by cloning the 4,955 bp ClaI-SalI
fragment of pHXB2D, containing most of the gag gene and
35  all of the pol gene, into the 4,150 bp ClaI-SalI fragment
of pHIVG5. The plasmid generated by this manipulation is
called pHIVG6.

Extraneous 3'-noncoding sequence was then
eliminated.  This was accomplished by cloning a 360 bp
AflII-BamHI PCR fragment, containing the 3'-end of the
pol gene, into the 8,030 bp AflII-BamHI fragment of
5  pHIVG6.  (This PCR fragment was generated from the
plasmid, pHXB2D, with the oligonucleotides, HIVP7 (SEQ ID
NO:122) and HIVP8 (SEQ ID NO:123)).  The plasmid
generated by this manipulation is called pHIVG7.

The I3L-promoted gag and pol genes were then
10  inserted into a canary pox insertion vector.  This was
accomplished by cloning the 4,360 bp partial BglII-BamHI
fragment of pHIVG7, containing the I3L-promoted gag and
pol genes, into the BamHI site of pVQH6CP3L.  The plasmid
generated by this manipulation is called pHIVGE14.

15          The H6-promoted HIV1 gp120 (+ transmembrane)
gene was then cloned into pHIVGE14.  This was
accomplished by cloning the 1,700 bp NruI-SmaI fragment
of pC5HIVMN120T, containing the gp120 (+ transmembrane)
gene, into the 11,400 bp NruI-SmaI fragment of pHIVGE14.
20  The resulting plasmid is called pHIVGE14T.

Most of the pol gene was then removed.  This
was accomplished by cloning a 540 bp ApaI-BamHI PCR
fragment, containing the 3'-end of the HIV1 protease
"gene", into the 10,000 bp ApaI-BamHI fragment of
25  pHIVGE14T.  (This PCR fragment was generated from the
plasmid, pHIVG7, with the oligonucleotides, HIVP5 (SEQ ID
NO:116) and HIVP37 (SEQ ID NO:180; 5'-
AAAGGATCCCCCGGGTTAAAAATTTAAAGTGCAACC-3')).  This
manipulation removes most of the pol gene, but leaves the
30  protease "gene" intact.  The plasmid generated by this
manipulation is called pHIV32.

pHIV32 is used in *in vitro* recombination
experiments with ALVAC as the rescuing virus.

**Example 48    -    GENERATION OF HIV1 GENES IN NYVAC**

35          Generation of NYVAC/HIV1 gag (+pro) and gp120
(+ transmembrane) Recombinant.  A plasmid, pHXB2D,
containing human immunodeficiency virus type 1 (HIV1)

120

cDNA sequence (IIIB) was obtained from Dr. R.C. Gallo
(NCI-NIH). The sequence encoding the 5'-end of the gag
gene was cloned between vaccinia virus tk flanking arms.
This was accomplished by cloning the 1,625 bp BglII
5   fragment of pHXB2D, containing the 5'-end of the gag
gene, into the 4,075 bp BglII fragment of pSD542VCVQ.
The plasmid generated by this manipulation is called
pHIVG2.

            The 3'-end of the gag gene was then cloned into
10  pHIVG2. This was accomplished by cloning a 280 bp ApaI-
BamHI PCR fragment, containing the 3'-end of the gag
gene, into the 5,620 bp ApaI-BamHI fragment of pHIVG2.
(This PCR fragment was generated from the plasmid,
pHXB2D, with the oligonucleotides, HIVP5 (SEQ ID NO:116)
15  and HIVP6 (SEQ ID NO:117)). The plasmid generated by
this manipulation is called pHIVG3.

            The I3L promoter was then cloned upstream of
the gag gene. This was accomplished by cloning the
oligonucleotides, HIVL17 (SEQ ID NO:118) and HIVL18 (SEQ
20  ID NO:119), encoding the vaccinia virus I3L promoter and
the 5'-end of the gag gene, into the 5,540 bp partial
BglII-ClaI fragment of pHIVG3. The plasmid generated by
this manipulation is called pHIVG4.

            The portion of the gag gene encoding p24, p2,
25  p7 and p6 was then eliminated. This was accomplished by
cloning the oligonucleotides, HIVL19 (SEQ ID NO:120) and
HIVL20 (SEQ ID NO:121), into the 4,450 bp partial PvuII-
BamHI fragment of pHIVG4. The plasmid generated by this
manipulation is called pHIVG5.

30          The remainder of the gag gene, as well as the
pol gene, was then cloned downstream of the p17 "gene".
This was accomplished by cloning the 4,955 bp ClaI-SalI
fragment of pHXB2D, containing most of the gag gene and
all of the pol gene, into the 4,150 bp ClaI-SalI fragment
35  of pHIVG5. The plasmid generated by this manipulation is
called pHIVG6.

121

Extraneous 3'-noncoding sequence was then
eliminated.  This was accomplished by cloning a 360 bp
AflII-BamHI PCR fragment, containing the 3'-end of the
pol gene, into the 8,030 bp AflII-BamHI fragment of
5    pHIVG6.  (This PCR fragment was generated from the
plasmid, pHXB2D, with the oligonucleotides, HIVP7 (SEQ ID
NO:122) and HIVP8 (SEQ ID NO:123)).  The plasmid
generated by this manipulation is called pHIVG7.

The I3L-promoted gag and pol genes were then
10   inserted into a canary pox insertion vector.  This was
accomplished by cloning the 4,360 bp partial BglII-BamHI
fragment of pHIVG7, containing the I3L-promoted gag and
pol genes, into the BamHI site of pVQH6CP3L.  The plasmid
generated by this manipulation is called pHIVGE14.

15          The H6-promoted HIV1 envelope "gp120" gene (MN)
was then inserted into pHIVGE14.  This was accomplished
by cloning the 1,600 bp NruI-NotI fragment of
pBSHIVMN120, containing the H6-promoted envelope "gp120"
gene, and the oligonucleotides, HIVL29 (SEQ ID NO:129)
20   and HIVL30 (SEQ ID NO:130), into the 11,500 bp NruI-XhoI
fragment of pHIVGE14.  The plasmid generated by this
manipulation is called pHIVGE15.

The H6-promoted HIV1 envelope "gp120" gene (MN)
and the I3L-promoted gag and pol genes (IIIB) were then
25   inserted into a vaccinia virus insertion vector.  This
was accomplished by cloning the 6,400 bp NotI-BamHI
fragment of pHIVGE15, containing the H6-promoted HIV1
envelope "gp120" gene (MN) and the I3L-promoted gag and
pol genes (IIIB), into the 4,000 bp NotI-BglII fragment
30   of pSD542 (defined in Example 15).  The plasmid generated
by this manipulation is called pHIVGE16.

The "gp120" gene was then replaced by the
"gp160" gene.  This was accomplished by cloning the 2,600
bp NruI-NotI fragment of pH6HMNE (defined in Example 10),
35   containing the entire HIV1 envelope gene (MN), into the
8,000 bp partial NruI-NotI fragment of pHIVGE16.  The

plasmid generated by this manipulation is called
pHIVGE19.

The part of the env gene encoding gp41 was then
replaced with the env transmembrane region. This was
5  accomplished by cloning the 1,700 bp NruI-NotI fragment
of pBSHIVMN120T, containing the H6-promoted gp120 (+
transmembrane) gene, into the 8,500 bp partial NruI-NotI
fragment of pHIVGE19. The resulting plasmid is called
pHIVGE19T.

10     Most of the pol gene was then removed. This
was accomplished by cloning a 540 bp ApaI-SmaI PCR
fragment, containing the 3'-end of the HIV1 protease
"gene", into the 7,000 bp ApaI-SmaI fragment of
pHIVGE19T. (This PCR fragment was generated from the
15  plasmid, pHIVG7, with the oligonucleotides, HIVP5 (SEQ ID
NO:116) and HIVP37 (SEQ ID NO:180)). This manipulation
removes most of the pol gene, but leaves the protease
"gene" intact. The plasmid generated by this
manipulation is called pHIV33.

20     pHIV33 is used in *in vitro* recombination
experiments with NYVAC as the rescuing virus.
Example 49   -   GENERATION OF HIV2 GENES IN ALVAC
         Generation of ALVAC/HIV2 gag/pol Recombinant.
A plasmid, pBSH6HIV2ENV (defined in Example 4), contains
25  the H6-promoted human immunodeficiency virus type 2
(HIV2) env gene. The H6-promoted env gene from this
plasmid was cloned between canary pox flanking arms.
This was accomplished by cloning the 2,700 bp XhoI-SacII
fragment of pBSH6HIV2ENV and the oligonucleotides, HIV2L4
30  (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the
XhoI-EcoRI site of pC6L (defined in Example 50). The
plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned
into pHIV23. This was accomplished by cloning the 4,450
35  bp XmaI-NotI fragment of pHIV22, containing the I3L-
promoted HIV2 gag and pol genes, and the
oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ

ID NO:132), into the 7,000 bp XmaI-XhoI fragment of
pHIV23. The plasmid generated by this manipulation is
called pHIV25.

The env gene was then removed. This was
5    accomplished by cloning the oligonucleotides, HIV2L8 (SEQ
ID NO:181; 5'-CGATAAACCGC-3') and HIV2L9 (SEQ ID NO:182;
5'-GGTTTAT-3'), into the 8,800 bp partial ClaI-SstII
fragment of pHIV25. The plasmid generated by this
manipulation is called pHIV27.

10   pHIV27 was used in in vitro recombination
experiments with ALVAC as the rescuing virus to yield
vCP190.

Generation of ALVAC/HIV2 env (gp160)
Recombinant. A plasmid, pBSH6HIV2ENV (defined in Example
15   4), contains the H6-promoted human immunodeficiency virus
type 2 (HIV2) env gene. The H6-promoted env gene from
this plasmid was cloned between canary pox flanking arms.
This was accomplished by cloning the 2,700 bp XhoI-SacII
fragment of pBSH6HIV2ENV and the oligonucleotides, HIV2L4
20   (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the
XhoI-EcoRI site of pC6L (defined in Example 50). The
plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned
into pHIV23. This was accomplished by cloning the 4,450
25   bp XmaI-NotI fragment of pHIV22, containing the I3L-
promoted HIV2 gag and pol genes, and the
oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ
ID NO:132), into the 7,000 bp XmaI-XhoI fragment of
pHIV23. The plasmid generated by .iis manipulation is
30   called pHIV25.

The gag and pol genes were then removed. This
was accomplished by cloning the oligonucleotides, HIV2L10
(SEQ ID NO:183; 5'-GGGAAAG-3') and HIV2L11 (SEQ ID
NO:184; 5'- GATCCTTTCCC-3'), into the 7,000 bp partial
35   BamHI-SmaI fragment of pHIV25. The plasmid generated by
this manipulation is called pHIV28.

124

pHIV28 was used in *in vitro* recombination experiments with ALVAC as the rescuing virus to yield vCP188.

Generation of ALVAC/HIV2 gp120 Recombinant. A

5    plasmid, pBSH6HIV2ENV, contains the H6-promoted human immunodeficiency virus type 2 (HIV2) env gene. The H6-promoted env gene from this plasmid was cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp XhoI-SacII fragment of pBSH6HIV2ENV

10   (defined in Example 4) and the oligonucleotides, HIV2L4 (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the XhoI-EcoRI site of pC6L. The plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned

15   into pHIV23. This was accomplished by cloning the 4,450 bp XmaI-NotI fragment of pHIV22, containing the I3L-promoted HIV2 gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ ID NO:132), into the 7,000 bp XmaI-XhoI fragment of

20   pHIV23. The plasmid generated by this manipulation is called pHIV25.

The gag and pol genes were then removed. This was accomplished by cloning the oligonucleotides, HIV2L10 (SEQ ID NO:183) and HIV2L11 (SEQ ID NO:184), into the

25   7,000 bp partial BamHI-SmaI fragment of pHIV25. The plasmid generated by this manipulation is called pHIV28.

The part of the env gene encoding gp41 was then removed. This was accomplished by cloning the 360 bp PstI-XbaI fragment of pBSHIV2120B, containing the 3′-end

30   of the gp120 gene, and the oligonucleotides, HIV2L12 (SEQ ID NO:185; 5′-CTAGAAAACCGC-3′) and HIV2L13 (SEQ ID NO:186; 5′-GGTTTT-3′), into the 5,600 bp PstI-SstII fragment of pHIV28. The plasmid generated by this manipulation is called pHIV29.

35   pHIV29 is used in *in vitro* recombination experiments with ALVAC as the rescuing virus.

### Generation of ALVAC/HIV2 gag/pol and gp120

Recombinant. A plasmid, pBSH6HIV2ENV (defined in Example
4), contains the H6-promoted human immunodeficiency virus
type 2 (HIV2) env gene. The H6-promoted env gene from
5 this plasmid was cloned between canary pox flanking arms.
This was accomplished by cloning the 2,700 bp XhoI-SacII
fragment of pBSH6HIV2ENV and the oligonucleotides, HIV2L4
(SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the
XhoI-EcoRI site of pC6L (defined in Example 50). The
10 plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned
into pHIV23. This was accomplished by cloning the 4,450
bp XmaI-NotI fragment of pHIV22, containing the I3L-
promoted HIV2 gag and pol genes, and the
15 oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ
ID NO:132), into the 7,000 bp XmaI-XhoI fragment of
pHIV23. The plasmid generated by this manipulation is
called pHIV25.

The gag and pol genes were then removed. This
20 was accomplished by cloning the oligonucleotides, HIV2L10
(SEQ ID NO:183) and HIV2L11 (SEQ ID NO:184), into the
7,000 bp partial BamHI-SmaI fragment of pHIV25. The
plasmid generated by this manipulation is called pHIV28.

The part of the env gene encoding gp41 was then
25 removed. This was accomplished by cloning the 360 bp
PstI-XbaI fragment of pBSHIV2120B, containing the 3'-end
of the gp120 gene, and the oligonucleotides, HIV2L12 (SEQ
ID NO:185) and HIV2L13 (SEQ ID NO:186), into the 5,600 bp
PstI-SstII fragment of pHIV28. The plasmid generated by
30 this manipulation is called pHIV29.

The gp120 gene was then cloned into pHIV25.
This was accomplished by cloning the 1,550 bp NruI-SacII
fragment of pHIV29, containing the H6-promoted gp120
gene, into the 9,000 bp NruI-SacII fragment of pHIV25.
35 The plasmid generated by this manipulation is called
pHIV30.

pHIV30 is used in *in vitro* recombination experiments with ALVAC as the rescuing virus.

<u>Generation of ALVAC/HIV2 gp120 (+ transmembrane) Recombinant</u>. A plasmid, pBSH6HIV2ENV

5 (defined in Example 4), contains the H6-promoted human immunodeficiency virus type 2 (HIV2) env gene. The H6-promoted env gene from this plasmid was cloned between canary pox flanking arms. This was accomplished by cloning the 2,700 bp <u>Xho</u>I-<u>Sac</u>II fragment of pBSH6HIV2ENV

10 and the oligonucleotides, HIV2L4 (SEQ ID NO:176) and HIV2L5 (SEQ ID NO:177), into the <u>Xho</u>I-<u>Eco</u>RI site of pC6L (defined in Example 50). The plasmid generated by this manipulation is called pHIV23.

The HIV2 gag and pol genes were then cloned

15 into pHIV23. This was accomplished by cloning the 4,450 bp <u>Xma</u>I-<u>Not</u>I fragment of pHIV22, containing the I3L-promoted HIV2 gag and pol genes, and the oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ ID NO:132), into the 7,000 bp <u>Xma</u>I-<u>Xho</u>I fragment of

20 pHIV23. The plasmid generated by this manipulation is called pHIV25.

The gag and pol genes were then removed. This was accomplished by cloning the oligonucleotides, HIV2L10 (SEQ ID NO:183) and HIV2L11 (SEQ ID NO:184), into the

25 7,000 bp partial <u>Bam</u>HI-<u>Sma</u>I fragment of pHIV25. The plasmid generated by this manipulation is called pHIV28.

The part of the env gene encoding gp41 was then removed. This was accomplished by cloning the 360 bp <u>Pst</u>I-<u>Xba</u>I fragment of pBSHIV2120B, containing the 3'-end

30 of the gp120 gene, and the oligonucleotides, HIV2L12 (SEQ ID NO:185) and HIV2L13 (SEQ ID NO:186), into the 5,600 bp <u>Pst</u>I-<u>Sst</u>II fragment of pHIV28. The plasmid generated by this manipulation is called pHIV29.

The HIV1 env transmembrane region was then

35 cloned onto the end of the gp120 gene. This was accomplished by cloning the 500 bp <u>Eco</u>RI fragment of pHIV31, containing the 3'-end of the gp120 gene and the

env transmembrane region, into the 5,600 bp EcoRI
fragment of pHIV29. (pHIV31 was derived by cloning a 500
bp PstI-XbaI PCR fragment, containing the 3'-end of the
gp120 gene and the env transmembrane region, into the
5     PstI-XbaI site of pIBI25.  This PCR fragment was
generated from the PCR fragment, PCRTM1, and the
oligonucleotides, HIVTM1 (SEQ ID NO:107) and HIVTM2 (SEQ
ID NO:108), with the oligonucleotides, HIV2P14 (SEQ ID
NO:187; 5'-CAGAAGTAGCATATATGT-3') and HIVTM3 (SEQ ID
10    NO:109).  PCRTM1 was generated from the plasmid, pHIV28,
with the oligonucleotides, HIV2P14 (SEQ ID NO:187) and
HIV2P15 (SEQ ID NO:188; 5'-
GCCTCCTACTATCATTATGAATAATCTCTTATGTCTCCCTGGAGC-3')).  The
plasmid generated by this manipulation is called pHIV34.
15         pHIV34 is used in *in vitro* recombination
experiments with ALVAC as the rescuing virus.

            Generation of ALVAC/HIV2 gag/pol and gp120 (+
transmembrane) Recombinant.  A plasmid, pBSH6HIV2ENV
(defined in Example 4), contains the H6-promoted human
20    immunodeficiency virus type 2 (HIV2) env gene.  The H6-
promoted env gene from this plasmid was cloned between
canary pox flanking arms.  This was accomplished by
cloning the 2,700 bp XhoI-SacII fragment of pBSH6HIV2ENV
and the oligonucleotides, HIV2L4 (SEQ ID NO:176) and
25    HIV2L5 (SEQ ID NO:177), into the XhoI-EcoRI site of pC6L
(defined in Example 50).  The plasmid generated by this
manipulation is called pHIV23.

            The HIV2 gag and pol genes were then cloned
into pHIV23.  This was accomplished by cloning the 4,450
30    bp XmaI-NotI fragment of pHIV22, containing the I3L-
promoted HIV2 gag and pol genes, and the
oligonucleotides, HIV2L6 (SEQ ID NO:131) and HIV2L7 (SEQ
ID NO:132), into the 7,000 bp XmaI-XhoI fragment of
pHIV23.  The plasmid generated by this manipulation is
35    called pHIV25.

            The gag and pol genes were then removed.  This
was accomplished by cloning the oligonucleotides, HIV2L10

(SEQ ID NO:183) and HIV2L11 (SEQ ID NO:184), into the
7,000 bp partial BamHI-SmaI fragment of pHIV25. The
plasmid generated by this manipulation is called pHIV28.

The part of the env gene encoding gp41 was then
5  removed. This was accomplished by cloning the 360 bp
PstI-XbaI fragment of pBSHIV2120B (defined in Example 4),
containing the 3'-end of the gp120 gene, and the
oligonucleotides, HIV2L12 (SEQ ID NO:185) and HIV2L13
(SEQ ID NO:186), into the 5,600 bp PstI-SstII fragment of
10 pHIV28. The plasmid generated by this manipulation is
called pHIV29.

The HIV1 env transmembrane region was then
cloned onto the end of the gp120 gene. This was
accomplished by cloning the 500 bp EcoRI fragment of
15 pHIV31, containing the 3'-end of the gp120 gene and the
env transmembrane region, into the 5,600 bp EcoRI
fragment of pHIV29. pHIV31 was derived by cloning a 500
bp PstI-XbaI PCR fragment, containing the 3'-end of the
gp120 gene and the env transmembrane region, into the
20 PstI-XbaI site of pIBI25 (IBI, New Haven, CT). This PCR
fragment was generated from the PCR fragment, PCRTM1, and
the oligonucleotides, HIVTM1 (SEQ ID NO:107) and HIVTM2
(SEQ ID NO:108), with the oligonucleotides, HIV2P14 (SEQ
ID NO:187) and HIVTM3 (SEQ ID NO:109). PCRTM1 was
25 generated from the plasmid, pHIV28, with the
oligonucleotides, HIV2P14 (SEQ ID NO:187) and HIV2P15
(SEQ ID NO:188). The plasmid generated by this
manipulation is called pHIV34.

The gp120 (+ transmembrane) gene was then
30 cloned into a plasmid containing the HIV2 gag/pol genes.
This was accomplished by cloning the 2,700 bp ClaI
fragment of pHIV34, containing the H6-promoted gp120 (+
transmembrane) gene, into the 6,800 bp ClaI fragment of
pHIV30. (pHIV30 was derived by cloning the 1,550 bp
35 NruI-SacII fragment of pHIV29, containing the H6-promoted
gp120 gene, into the 9,000 bp NruI-SacII fragment of

pHIV25.) The plasmid generated by this manipulation is called pHIV35.

pHIV35 is used in *in vitro* recombination experiments with ALVAC as the rescuing virus.

5   **Example 50 - EXPRESSION OF TWO FUSION PEPTIDES CONTAINING THE p24 EPITOPE OF HIV-1 *gag* FUSED TO THE T1 AND V3 LOOP EPITOPES OF HIV-1 *env* WITH AND WITHOUT THE SIGNAL DOMAIN FROM HIV-1 *env***

10          Two expression cassettes were generated by a series of polymerase chain reactions described below. These cassettes differ in that one version encodes the signal sequences of HIV-1 *env* fused to the epitopes whereas the other does not.

15          The version of the fusion peptide with the signal is preceded by the 51 amino acid N-terminal portion of HIV-1 (IIIB) *env*, residues 1-50 (plus initiating Met) based on Ratner et al. (1985) followed by a cleavable linker region. The amino acid sequence of

20  this region is (SEQ ID NO:189) MKEQKTVAMRVKEKYQHLWRWGWRWGTMLLGMLMICSATEKLWVTVYYGVP-PFRK. Both versions of the fusion peptide, contain an amino acid sequence based on the defined T-cell epitopes of p24, the V3 loop (MN), and T1 of HIV-1 (MN) *env*. The

25  peptide is designed such that the epitopes are separated from each other and the signal where present by the sequence (SEQ ID NO:189) PPFRK. The sequence of this region of the peptide is (SEQ ID NO:190) [signal-PFRK]- GPKEPFRDYVDRFYK-PPFRK-

30  VQINCTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAH CNISRAK-PPFR- KQIINMWQEVEKAMYA. In the version lacking the signal sequence, the region indicated by [signal-PFRK] is replaced by an initiating methionine only.

          For the cassette with the signal, the H6

35  promoter and signal were derived by PCR from plasmid pBST1T2TH4.1 (described above) using primers H6PCR1 (SEQ ID NO:157; 5'- ACTACTAAGCTTCTTTATTCTATACTTAAAAAGTG -3')

130

and SIGPCR24 (SEQ ID NO:191; 5'-
AGGTCCCTTCCTGAATGGAGGTACCCCATAATAGACTG-3'). The p24
epitope was fused to the signal sequence by PCR
amplification of this 307 bp PCR-derived fragment and

5   oligonucleotides P24A (SEQ ID NO:192; 5'-
CCATTCAGGAAGGGACCTAAAGAACCTTTTAGAGATTATGTAGATAGATTTTATAAA
CCACCTTTTAGAAAA-3') and P24B (SEQ ID NO:193; 5'-
TTTTCTAAAAGGTGGTTTATAAAATCTATCTACATAATCTCTAAAAGGTTCTTTAGG
TCCCTTCCTGAATGG-3') using primers H6PCR1 and P24PCR (SEQ

10  ID NO:194; 5'-
GTACAATTAATTTGTACTTTTCTAAAAGGTGGTTTATAAAATC-3'). This
377 bp PCR-derived fragment consists of the H6 promoter
coupled to coding sequences for the signal, the p24
epitope and the first 6 amino acids of the V3 loop.

15          For the cassette without the signal, the H6
promoter were derived by PCR from plasmid pH6T2
(described above) using primers H6PCR1 (SEQ ID NO:157)
and H6P24 (SEQ ID NO:195; 5'-
GGTGGTTTATAAAATCTATCTACATAATCTCTAAAAGGTTCTTTAGGTCCCATTACG

20  ATACAAACTTAACGG-3'). The p24 epitope was fused to the
promoter by PCR amplification of this 187 bp PCR-derived
fragment and oligonucleotides P24A (SEQ ID NO:192) and
P24B (SEQ ID NO:193) using primers H6PCR1 (SEQ ID NO:157)
and P24PCR (SEQ ID NO:194). This 214 bp PCR-derived

25  fragment consists of the H6 promoter coupled to coding
sequences for the p24 epitope and the first 6 amino acids
of the V3 loop.

          Coding sequences for the V3 loop region were
derived by PCR amplification of the SmaI fragment from

30  plasmid pHIVGE16EV (described above) using primers V3PCR1
(SEQ ID NO:196; 5'-AAACCACCTTTTAGAAAAGTACAAATTAATTGTAC-
3') and V3PCR2 (SEQ ID NO:197; 5'-
CTGCTTACGGAACGGTGGTTTTGCTCTACTAATGTTACAATG-3'). The T1
epitope was joined to the coding region for the V3 loop

35  by PCR amplification of this 171 bp PCR-derived fragment
and oligonucleotides MNT1A (SEQ ID NO:198; 5'-
CCACCGTTCCGTAAGCAGATAATAAACATGTGGCAAGAAGTAGAAAAAGCTATGTAT

GCTTAA-3') and MNT1B (SEQ ID NO:199; 5'-
TTAAGCATACATAGCTTTTTCTACTTCTTGCCACATGTTTATTATCTGCTTACGGAA
CGGTGG-3') as template using primers V3PCR1 (SEQ ID
NO:196) and T1MNPCR (SEQ ID NO:200; 5'-

5   TCATCAAAGCTTCTCGAGAAAAATTAAGCATACATAGCTTTTTC-3'). This
239 bp PCR-derived fragment consists of the last codon of
p24 epitope, the V3 loop, ant the T1 epitope followed 3'
by an early transcription termination signal (TTTTTNT)
and XhoI and HindIII sites.

10          For the cassette with the signal, the promoter,
signal and p24 epitope coding sequences were joined to
the coding region for the V3 loop/T1 epitope by PCR
amplification of the 377 bp and 239 bp PCR-derived
fragments using primers H6PCR1 (SEQ ID NO:157) and

15  T1MNPCR (SEQ ID NO:200). Following digestion of this 581
bp PCR-derived fragment with HindIII, a fragment of 563
bp was isolated from an agarose gel, ligated to similarly
digested pBS (Stratagene, La Jolla, CA) creating plasmid
pMNT1P24. The insert was verified by nucleotide sequence

20  analysis.

            For the cassette without the signal, the
promoter and p24 coding sequences were joined to the
coding region for the V3 loop/T1 epitope by PCR
amplification of the 171 bp and 239 bp PCR-derived

25  fragments using primers H6PCR1 (SEQ ID NO:157) and
T1MNPCR (SEQ ID NO:200). Following digestion of this 418
bp PCR-derived fragment with HindIII, a fragment of 400
bp was isolated from an agarose gel, ligated to similarly
digested pBS (Stratagene, La Jolla, CA) creating plasmid

30  pMN24EV3T1NSA. The insert was verified by nucleotide
sequence analysis.

            A C6 insertion vector containing 370 bp
upstream of C6, polylinker containing SmaI, PstI, XhoI,
and EcoRI sites, and 1156 bp of downstream sequence was

35  derived in the following manner. The 0.4 kb upstream
sequence was generated by PCR amplification of a cosmid
clone derived from purified genomic canarypox DNA using

132

oligonucleotides C6A1SG (SEQ ID NO:201;

5'- ATCATCGAGCTCGCGGCCGCCTATCAAAAGTCTTAATGAGTT -3') and

C6B1SG (SEQ ID NO:202;

5'- GAATTCCTCGAGCTGCAGCCCGGGTTTTTATAGCTAATTAGTCATTTTTTCGT

5  AAGTAAGTATTTTTATTTAA -3'). The 1.2 kb downstream arm was

generated by PCR amplification of the same template using

oligonucleotides C6C1SG (SEQ ID NO:203;

5'- CCCGGGCTGCAGCTCGAGGAATTCTTTTTATTGATTAACTAGTCAAATGAGTA

TATATAATTGAAAAAGTAA -3') and C6D1SG (SEQ ID NO:204;

10 5'- GATGATGGTACCTTCATAAATACAAGTTTGATTAAACTTAAGTTG -3').

These fragments were fused by a third PCR employing gel

purified 0.4 and 1.2 kb fragments as template for primers

C6A1SG (SEQ ID NO:201) and C6D1SG (SEQ ID NO:204). The

resulting 1.6 kb fragment was isolated from an agarose

15 gel, digested with SacI and KpnI and ligated to similarly

digested pBS (Stratagene, La Jolla, CA) generating C6

insertion plasmid pC6L.

Both expression cassettes were excised by

PstI/XhoI digestion of pMNT1P24 and pMN24EV3T1NSA,

20 isolated from agarose gels and ligated separately to

similarly digested pC6L creating plasmids pC6P24FS and

pC6P24FNS respectively, for recombination into the C6

locus of ALVAC. The resulting recombinants are

designated vCP189 and vCP195, respectively.

25      BamHI/XhoI fragments from pMNT1P24 and

pMN24EV3T1NSA were ligated to similarly digested pSD550VC

(defined in Example 33) creating pI4P24FS and pI4P24FNS

for recombination into the I4 locus of NYVAC generating

vP1117 and vP1110, respectively.

30      Expression of Tetanus Toxin Fragment C in

Poxviruses Expressing HIV-1 Proteins. It has been

proposed that the addition of various Th epitopes from

homologous (Good et al., 1987) and heterologous proteins

(Francis et al., 1987) may be capable of recruiting T-

35 cell help for specific B cell responses to synthetic

peptide vaccines. In an effort to elicit enhanced immune

responses to HIV-1 antigens, various T-cell epitopes

derived from HIV-1 have been incorporated into
recombinant poxviruses. To further pursue this strategy,
tetanus toxin fragment C, which contains other known
human T-helper cell epitopes (Ho et al., 1990), will be

5   co-expressed with HIV-1 antigens in an ALVAC recombinant.
The presence of these epitopes from tetanus toxin may
enhance the immune response against HIV-1 by providing
nonspecific T-cell help.

PstI/SmaI digested pC6P24FS (described above)

10  was modified by ligating the PstI/SmaI fragment from
plasmid pVQ42KTH4.1 (described above) creating
pC6P24FSVQ. This plasmid was digested with NruI within
the H6 promoter and XhoI at the 3' end and ligated to a
1.4 kb fragment isolated from similarly digested pH6TETC

15  (described above). The resulting plasmid, pC6VQTETC, was
confirmed by restriction digestion and nucleotide
sequence analysis of the regions surrounding the cloning
sites. Following confirmation pC6VQTETC was employed in
recombination experiments with vCP112, vCP125 and vCP156.

20  **Example 51  -  PERIPHERAL BLOOD MONONUCLEAR CELL STUDIES**
                  **WITH NYVAC/HIV-1 AND ALVAC/HIV-1**
                  **RECOMBINANTS**

While broad issues concerning the
immunogenicity of NYVAC/HIV-1 and ALVAC/HIV-1 constructs

25  in man are best addressed within the context of clinical
trials, a number of relevant insights have already been
gleaned by *in vitro* cellular response assays. A central
question regarding the use of these vaccine constructs in
man is their capacity to impact on cellular anti-HIV-1

30  reactivities, especially cytotoxic T-lymphocytes (CTL)
responses. Whether as a component of a preventative or a
therapeutic vaccine strategy, there are ample precedents
suggesting a beneficial role for CTL in a number of viral
infections in man (McMichael et al., 1983; Moss et al.,

35  1978; Borysiewicz et al., 1988). Although the precise
contribution of anti-HIV-1 CTL in preventing or
controlling viral infection remains to be elucidated,

studies by Letvin and co-workers using the SIV/macaque
animal model suggest that anti-SIV-1 CTL, especially gag-
specific CTL, may represent a major determinant
controlling disease progression (Letvin and King, 1990).
5  This coupled with the observations of numerous
investigators that anti-HIV-1 CTL activities can be
measured directly from fresh peripheral blood mononuclear
cells (PBMC) in a relatively high proportion of
asymptomatic patients (reviewed in Walker and Plata,
10 1990; Autran et al., 1991), support the contention that
elicitation of anti-HIV-1 CTL reactivities should be
included as a major goal of both preventative and
therapeutic vaccines.

To test the capacity of NYVAC/HIV-1 and
15 ALVAC/HIV-1 constructs to impact on relevant CTL
reactivities, a series of experiments were conducted
involving a cohort of HIV-1 infected patients devoid of
detectable direct anti-HIV-1 gp160 CTL reactivities.
PBMCs were obtained from these patients and a portion of
20 the PBMC were infected with either the fully replication-
competent vaccinia/gp 160 construct vPE16 (Walker et al.,
1987), or the replication-attenuated NYVAC/gp160 or
ALVAC/gp160 constructs. The acutely infected PBMC were
washed and used as stimulators for the remaining PBMC in
25 a 10-day in vitro stimulation protocol. Controls
included both unstimulated and control vector (i.e.
parental poxvirus minus HIV-1 genes) stimulated cultures.
Following the 10-day incubation in the absence of
exogenous IL-2, cells were washed and evaluated for CTL
30 activities against autologous B-lymphocyte cell lines
(BLCL) infected with either a control vaccinia virus,
vSC8, or the vaccinia/gp160 vPE16 construct.
Additionally, phenotypic depletion of CD8+ cells was
performed in parallel using magnetic microsphere sorting.
35 The results of these studies shown in Fig. 11 revealed
that whereas unstimulated or control vector-stimulated
cultures had no detectable CTL activity against HIV-1

gp160 targeted BLCL, cultures stimulated with any of the
HIV-1 gp160 constructs had a high level of anti-gp160
cytolytic activity, all of which was abolished following
removal of CD8+ cells and thereby consistent with the

5   appearance of CTL activities.  Perhaps the most
significant observation to come out of these studies was
the consistent finding that the magnitude of the CTL
response was greater in the NYVAC/gp160 and ALVAC/gp160-
stimulated cultures than in those which were stimulated

10  with the fully replication competent vPE16 infected PBMC.
It is not known whether this apparent enhancement in CTL
stimulation by NYVAC/gp160 and ALVAC/gp160 was due to
differences in expression by the recombinants as compared
to vPE16 or to the attenuation characteristics of the

15  NYVAC and ALVAC vectors.

Extensive flow cytometric analyses of the
various stimulated cultures was also performed in order
to more thoroughly identify the effector cell phenotype
which was elicited.  When compared to either unstimulated

20  or control vector stimulated cultures, generation of
anti-gp160 cytolytic reactivities generally resulted in a
decline in $CD3^+/CD4^+$ subpopulations and a compensatory
rise in $CD3^+/CD8^+$ cells.  Significant increases in
$CD3^+/CD25^+$, $CD3^+/HLA-DR^+$, $CD8^+/S6F1^+$, $CD8^+/CD38^+$, and

25  $CD3^+/CD69^+$ cells were also noted.  When the cytolytic
activities of each culture were plotted against increases
in particular cell subpopulations, a linear relationship
was found between cellular anti-gp160 reactivity and
increases in the $CD8^+/S6F1^+$ population.  This cellular

30  phenotype broadly defines CTL (Morinoto et al., 1987).
These early pre-clinical studies with cells
from HIV-1 infected patients clearly illustrate the
capacity of NYVAC and ALVAC/HIV-1 vectors to elicit
potent CTL activities from precursor populations which

35  are contained within the PBMC pool of patients.
Furthermore, these studies strongly exemplify the
potential clinical utility of these vectors in the

context of a therapeutic vaccine strategy. This could take the form of simple immunization with these vectors or could involve an *ex vivo* component of either cellular targeting and re-infusion or *ex vivo* CTL generation

5   followed by large scale adoptive transfer. In either case, the combined safety of the non-replicating vectors and their inherently strong cellular immunogenicity make them ideal candidates for immune-based therapy in man.

Having thus described in detail preferred

10  embodiments of the present invention, it is to be understood that the invention defined by the appended claims is not to be limited to the particular details set forth in the above description as many apparent variations thereof are possible without departing from

15  the spirit or scope of the present invention.

137

## REFERENCES

1. Autran, B., Plata, F. and Debre, P., J. AIDS, **4**, 361-367 (1991)

2. Behbehani, A.M., Microbiological Reviews **47**, 455-509 (1983).

3. Bertholet, C., Drillien, R. and Wittek, R., Proc. Natl. Acad. Sci. **82**, 2096-2100 (1985).

4. Borysiewicz, I., Graham, S., Hickling, J., Mason, P.D., and Sissons, J.P.G., Eur. J. Immunol. **18**, 269-275 (1988)

5. Chakrabarti, S., Robert-Guroff, M., Wong-Staal, F., Gallo, R.C., and Moss, B., Nature **320**, 535-537 (1986).

6. Cianciolo, G.J., Copeland T.D., Oroszlan S., and Snyderman, R., Science **230**, 453-456 (1985).

7. Clewell, D.B., and Helinski, D.R., Proc. Natl. Acad. Sci. USA **62**, 1159-1166 (1969).

8. Clewell, D.B., J. Bacteriol **110**, 667-676 (1972).

9. Colinas, R.J., Condit, R.C., and Paoletti, E., Virus Research **18**, 49-70 (1990).

10. Derosiers, R.C., M.S., Wyand, T. Kodama, T.J. Ringler, L.O. Arthur, P.K. Seghal, N.L. Letvin, N.W. King, and M.D. Daniel Proc. Natl. Acad. Sci. USA **86**, 6353-6357 (1989).

11. Dreyfuss, G., Adam, S. A., and Choi, Y.-D., Mol. Cell. Biol. **4**, 415-423 (1984).

12. Engelke, D. R., Hoener, P. A., and Collins, F. S., Proc. Natl. Acad. Sci. USA **85**, 544-548 (1988).

13. Franchini, G., Gurgo, C., Guo, H.-G., Gallo, R. C., Collati, E., Fargnoli, K. A., Hall, L. F., Wong-Staal, F., and Reitz, Jr., M. S., Nature (London) **328**, 539-543 (1987).

14. Franchini, G., Fargnoli, K. A., Giomnini, F., Jagodzinski, L., DeRossi, A., Bosch, M., Biberfield, G., Fenyo, E. M., Albert, J., Gallo, R. C., and Wong-Staal, F., Proc. Natl. Acad. Sci. USA **86**, 2433-2437 (1989).

15. Francis, M.J., Hastings, G.Z., Syred, A.D., McGinn, B., Brown, F., and Rowlands, D.J., Nature **330**, 168-170 (1987).

138

16. Gillard, S., Spehner, D., Drillien, R., and Kirn, A., Proc. Natl. Acad. Sci. USA **83**, 5573-5577 (1986).

17. Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E., Virology **179**, 247-266 (1990a).

18. Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E., Virology **179**, 517-563 (1990b).

19. Good, M.F., Maloy, W.L., Lunde, M.N., Margalit, H., Cornette, J.L., Smith, G.L., Moss, B., Miller, L.H., and Berzofsky, J.A., Science **235**, 1059-1062 (1987).

20. Guo, H.-G., Veronese, F., Tschachler, E., pal, R., Kalyanaraman, V.S., Gallo, R.C., and Reitz, Jr., M.S., Virology **174**, 217-214 (1990).

21. Guo, P., Goebel, S., Davis, S., Perkus, M. E., Taylor, J., Norton, E., Allen, G., Lanquet, B., Desmettre P., and Paoletti, E., J. Virol. **64**, 2399-2406 (1990).

22. Guo, P., Goebel, S., Davis, S., Perkus, M. E., Lanquet, B., Desmettre, P., Allen, G., and Paoletti, E., J. Virol. **63**, 4189-4198 (1989).

23. Gurgo, C., Guo, H.-G., Franchini, G., Aldovini, A., Collalti, E., Farrell, K., Wong-Staal, F., Gallo, R.C., and Reitz, M.S., Jr., Virology **164**, 531 (1988).

24. Haffar, O., Garrigues, J., Travis, B., Moran, P., Zarling, J., and Hu, S.-L., J. Virol. **64**, 2653-2659, (1990).

25. Ho, P.C., Mutch, D.A., Winkel, K.D., Saul, A.J., Jones, G.L., Doran, T.J., and Rzepczyk, C.M., Eur. J. Immunol. **20**, 477-483 (1990).

26. Hosmalin, A., Nara, P.L., Zweig, M., Lerche, N.W., Cease, K.B., Gard, E.A., Markham, P.D., Putney, S.D., Daniel, M.D., Desrosiers, R.C., and Berzofsky, J.A. J. Immunol. **146**, 1667-1673 (1991).

27. Hu, S.-L., Fultz, P., McClure, H., Eichberg, J., Thomas, E., Zarling, J., Singhal, M., Kosowski, S., Swenson, R., Anderson, D., and Todaro, G., Nature **328**, 721-723 (1987).

28. Hu, S.-L., Travis, B. M., Garrigues, J., Zarling, J. M., Eichberg, J. W. and Alpers, C. E., In Vaccine 90, eds. Chanock, R. M., Lerner, R. A., Brown, F., and Ginsberg, H., (Cold Spring Harbor Press, Cold Spring Harbor, New York) pp. 231-236 (1990).

139

29. Hu, S.-L., Kosowski, S., and Dalrymple, J., Nature
320, 535-537, (1986).

30. Hu, S.L., Zarling, J.M., Chinn, J., Travis, B.M.,
Moran, P.A., Sias, J., Kuller, L., Morton, W.R.,
Heidecker, G., and Benveniste, R.E., Proc. Natl.
Acad. Sci. USA 86, 7213 (1989).

31. Javeherian, K., Langlois, A.J. M$^c$Danal, C., Ross,
K.L., Eckler, L.I., Jellib, C.L., Profy, A.T.,
Rusche, J.R., Bolognesi, D.P., Putney, S.D., and
Matthews, T.J. Proc. Natl. Acad. Sci. USA 86, 6768-
6772 (1989).

32. Karacostas, V., Nagashima, K., Gonda, M.A., and
Moss, B. Proc. Natl. Acad. Sci. USA 86, 8964-8968
(1989).

33. Klasse, P. J., Pipkorn, R., and Blomberg, J., Proc.
Natl. Acad. Sci. USA 85, 5225-5229 (1988).

34. Knauf, V.C. and Nester, E.W., Plasmid 8, 45-54
(1982).

35. Kodama, T., Wooley, D.P., Naidu, Y.M., Kestler III,
H.W., Daniel, M.D., Li, Y., and Derisiers, R.C. J.
Virol. 63, 4709-4714 (1989).

36. Koff, W. C. and Fauci, A. S., AIDS 1, 5125-5129
(1989).

37. Koup, R.A., Sullivan, J.L., Levine, P.H., Brettler,
D., Mahr, A., Mazzara, G., M$^c$Kenzie, S., and
Panicali, D. Blood 73, 1909-1919 (1989).

38. Kunkel, T. A., Proc. Natl. Acad. Sci. USA 82, 488-
492 (1985).

39. Laemmli, U. K., Nature (London) 227, 680-685 (1970).

40. Lane, J.M., Ruben, F.L., Neff, J.M., and Millar,
J.D. New Eng. J. Med. 281, 1201-1208 (1969).

41. LaRosa, G. J., Davide, J. P., Weinhold, K.,
Waterbury, J. A.,   Profy, A. T., Lewis, J. A.,
Langlois, A. J., Dressman, G. R., Boswell, R. N.,
Shadduck, P., Holley, L. H., Karpus, M., Bolognesi,
D. P., Matthews, T. J., Emini, E. A., and Putney, S
.D., Science 249, 932-935 (1990).

42. Letvin, N.L., and King, N.W., J. AIDS, 3, 1023-40
(1990)

43. Mandecki, W., Proc. Natl. Acad. Sci. USA 83, 7177-
7182 (1986).

44. Maniatis, T., Fritsch, E. F., and Sambrook, J. In Molecular cloning: a laboratory manual, (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (1982).

45. McMichael, A.J., Gotch, F.M., Noble, G.R. and Beare, P.A.S., New Engl. J. Med. **309**, 13-17 (1983)

46. Michel, F., Hoffenbach, A., Langlade-Demoyen, P., Guy, B., Lecocq, J.-P., Wain-Hobson, S., Kieny, M.-P., and Plata, F., Eur. J. Immunology **18**, 1917 -1927 (1988).

47. Moss, D.J., Rickinson, A.B., and Pope, J.H., Int. J. Cancer **22**, 662-668 (1978)

48. Murphy-Corb, M., Martin, L. N., Davison-Fairburn, B., Montelaro, R. C., Miller, M., West, M., Ohkawa, S., Baskin, G. B., Zhang, J.-Y., Putney, S. D., Allison, A. C. and Eppstein, D. A., Science **246**, 1293-1297 (1989).

49. Nixon, D. F., Townsend, A. R. M., Elvin, J. G., Rizza, C. R., Gallwey, J., and McMichael, A. J., Nature **326**, 484-487 (1988).

50. Panicali, D., and Paoletti, E., Proc. Natl. Acad. Sci. USA **79**, 4927-4931 (1982).

51. Perkus, M. E., Piccini, A., Lipinskas, B. R., and Paoletti, E., Science **229**, 981-984 (1985).

52. Perkus, M. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Limbach, K., Norton, E. K., and Paoletti, E., Virology **179**, 276-286 (1990).

53. Perkus, M. E., Limbach K., and Paoletti, E., J. Virol. **63**, 3829-3836 (1989).

54. Piccini, A., Perkus, M. E., and Paoletti, E., Methods in Enzymology **153**, 545-563 (1987).

55. Plata, F., Autran, B., Martins, L. P., Wain-Hobson, S., Raphael, M., Mayaud, C., Denis, M., Guillon, J.-M., Debre, P., Nature **328**, 348-351 (1987).

56. Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Rafalski, J.A., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway, S.R.Jr., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C., and Wong-Staal, F., Nature **313**, 277 (1985).

141

57.  Ratner, L., Haseltine, W., Patarca, R., Livak, K.
     J., Starcich, B., Josephs, S. F., Doran, E. R.,
     Rafalski, J. A., Whitehorn, E. A., Baumeister, K.,
     Ivanoff, L., Petteway, Jr., S. R., Pearson, M. L.,
 5   Lautenberger, J. A., Papas, T. S., Ghrayeb, J.,
     Chang, N. T., Gallo., R. C., and Wong-Staal F.,
     Nature **313**, 277-284 (1985).

58.  Rautmann, G., Kieny, M.-P., Brandely, R., Dott, K.,
 10  Girard, M. Montagnier, L., and Lecocg, J.-P. AIDS
     Research and Human Retroviruses **5**, 147-157 (1989).

59.  Riviere Y., Tanneau-Salvadori, F., Regnault, A.,
     Lopez, O., Sansonetti, P., Guy, B., Kieny, M.-P.,
 15  Fournel, J.-J., and Montagnier, L., J. Virol. **63**,
     2270-2277 (1989).

60.  Ruegg, C. L., Monell, C. R., and Strand, M., J.
     Virol. **63**, 3250-3256 (1989a).
 20
61.  Ruegg, C. L., Monell, C. R., and Strand, M., J.
     Virol. **63**, 3257-3260 (1989b).

62.  Sanchez-Pascador, R., Power, M.D., Barr, P.J.,
 25  Steimer, K.S., Stampien, M.M., Brown-Shimer, S.L.,
     Gee, W., Renard, A., Randolph, A., Levy, J.A., Dina,
     D., and Luciw, P.A., Science **227**, 484-492 (1985).

63.  Sanger, F., Nickeln, S., and Coulson, A. R., Proc.
 30  Natl. Acad. Sci. **74**, 5463-5467 (1977).

64.  Schmaljohn, C.S., Jennings, G.B., Hay, J.,
     Dalrymple, J.M., Virology **155**, 633-43 (1986).

 35  65.  Schmitt, J.F.C., and Stunnenberg, H.G., J. Virol.
     **62**, 1889-1897 (1988).

66.  Shaffermann, A., Lennox, J., Grosfeld, H., Sadoff,
     J., Redfield, R. R., and Burke, D. S., AIDS Research and
 40  Human Retroviruses **5**, 33-39 (1989).

67.  Shapira, S. K., Chou, J., Richaud, F. V., and
     Casadaban, M. J., Gene **25**, 71-82 (1983).

 45  68.  Shioda, T. and H. Shibuta Virology **175**, 139-148
     (1990).

69.  Starcich et al., Cell **45**, 637-648 (1986).

 50  70.  Tabor, S., and Richardson, C. C., Proc. Natl. Acad.
     Sci. USA **84**, 4767-4771 (1987).

71.  Tartaglia, J. and Paoletti, E., _In_ Immunochemistry
     of viruses, II, eds. van Regenmortel, M. H. V. and
 55  Neurath, A. R., (Elsevier Science Publishers B.V.,
     Amsterdam) pp. 125 (1990).

72. Tartaglia, J., Winslow, J., Goebel, S., Johnson, G.P., Taylor, J., and Paoletti, E. J. Gen. Virol. **71**, 1517-1524 (1990).

73. Tartaglia, J. Perkus, M.E., Taylor, J. Norton, E.K., Audonnet, J.C., Cox, W.I., Davis, S.W., VanderHoven, J., Meignier, B., Riviere, M., Languet, B. and Paoletti, E., Virology, **188** (1992).

74. Taylor, J., Weinberg, R., Languet, B., Desmettre, P., and Paoletti, E., Vaccine **6**, 497-503 (1988a).

75. Taylor, J., Weinberg, R., Kawaoka, Y., Webster, R. G., and Paoletti, E., Vaccine **6**, 504-508 (1988b).

76. Taylor, J., Weinberg, R., Tartaglia, J., Richardson, C., Alkhatib, G., Briedis, D., Appel, M., Norton, E., and Paoletti, E., Virology **187**, 321-328 (1992).

77. Vos, J.C., and Stunnenberg, H.G., EMBO J. 7, 3487-3492 (1988).

78. Walker, B. D., Flexner, C., Paradis, T. J., Fuller, T. C., Hirsch, M. S., Schooley, R. T. and Moss, B., Science **240**, 64-66 (1988).

79. Walker, B. D., Chakrabarti, S., Moss, B., Paradi, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S., and Schooley, R. T., Nature **328**, 345-348 (1987).

80. Walker, B. D., Flexner, C., Birch-Limberger, K., Fisher, L., Paradis, T. J., Aldovini, A., Young, R., Moss, B., and Schooley, R. T., Proc. Natl. Acad. Sci. **86**, 9514-9519 (1989).

81. Walker, B.D. and Plata, F., AIDS **4**, 177-184 (1990)

82. Weiss, R. A., Clapham, P. R., Cheingsong-Popov, R., Dalgleish, G., Carne, C. A., Weller, I. V., and Tedder, R. S., Nature **316**, 69-72 (1985).

83. Yuen, L., and Moss, B., Proc. Natl. Acad. Sci. USA **84**, 6417-6421 (1987).

84. Zagury, D., Bernard, J., Cheynier, R., Desportes, I., Leonard, R., Fouchard, I., Reveil, B., Ittele, D., Lurhuma, Z., Mbayo, K., Wane, J., Salaun, J.-J., Goussard, B., Dechazal, L., Burny, A., Nara, P. and Gallo, R. C., Nature **332**, 728-731 (1988).

85. Zarling, J. M., Morton, W., Moran, P. A., McClure, J., Kosowski, S. G., and Hu, S.-L., Nature **323**, 344-346 (1986).

143

**WHAT IS CLAIMED IS:**

        1..   A modified recombinant virus, said
modified recombinant virus having nonessential virus-
encoded genetic functions inactivated therein so that the
5  virus has attenuated virulence, and said modified
recombinant virus comprising DNA from an immunodeficiency
virus in a nonessential region of the recombinant virus
genome.

        2.   A virus as claimed in claim 1, wherein
10  said virus is a poxvirus.

        3.   A virus as claimed in claim 2, wherein
said poxvirus is vaccinia virus.

        4.   A virus as claimed in claim 3, wherein the
genetic functions are inactivated by deleting an open
15  reading frame encoding a virulence factor.

        5.   A virus as claimed in claim 3, wherein the
genetic functions are inactivated by insertional
inactivation of an open reading frame encoding a
virulence factor.

20        6.   A virus as claimed in claim 4, wherein the
open reading frame is selected from the group consisting
of J2R, B13R + B14R, A26L, A56R, C7L – K1L, I4L and
combinations thereof.

        7.   A virus as claimed in claim 5, wherein the
25  open reading frame is selected from the group consisting
of J2R, B13R + B14R, A26L, A56R, C7L – K1L, I4L and
combinations thereof.

        8.   A virus as claimed in claim 4 wherein the
nonessential region of the recombinant virus genome where
30  the DNA from the immunodeficiency virus is located is
selected from the group consisting of J2R, B13R + B14R,
A26L, A56R, C7L-K1L, I4L and combinations thereof.

        9.   A virus as claimed in claim 5 wherein the
nonessential region of the recombinant poxvirus genome
35  where the DNA from the immunodeficiency virus is located
is selected from the group consisting of J2R, B13 + B14,
A26L, A56R, C7L-K1L, I4L and combinations thereof.

10. A virus as claimed in claim 3, wherein the immunodeficiency virus is human immunodeficiency virus.

11. A virus as claimed in claim 10 which is vP911, vP921, vP878, vP939, vP940, vP920, vP922, vP1008, 5 vP1004, vP1020, vP1078, vP994, vP1035, vP969, vP989, vP991, vP990, vP970, vP973, vP971, vP979, vP978, vP988, vP1009, vP1062, vP1060, vP1061, vP1084, vP1045, vP1047 or vP1044.

12. A virus as claimed in claim 3, wherein the 10 immunodeficiency virus is simian immunodeficiency virus.

13. A virus as claimed in claim 12 which is vP873, vP948, vP943, vP942, vP952, vP1042, vP1071 or vP1050.

14. A recombinant avipox virus having 15 attenutated virulence and containing therein DNA from an immunodeficiency virus in a nonessential region of the avipox virus genome.

15. A recombinant avipox virus as claimed in claim 14, wherein the immunodeficiency virus is human 20 immunodeficiency virus.

16. A recombinant avipox virus as claimed in claim 15, wherein the avipox virus is canarypox.

17. A recombinant avipox virus as claimed in claim 15, wherein the avipox virus is fowlpox.

25          18. A human immunodeficiency virus recombinant canarypox virus as claimed in claim 14 which is vCP95, vCP112, vCP60, vCP61, vcP125, vCP124, vCP126, vCP144, vCP120, vCP138, vCP117, vCP130, vCP152, vCP155, vCP156, vCP146, vCP148, vCP154, vCP168, vCP153 or vCP172.

30          19. A human immunodeficiency virus recombinant fowlpox virus as claimed in claim 17 which is vFP62, vFP63, or vFP174.

20. A vaccine for inducing an immunological response in a host animal inoculated with said vaccine, 35 said vaccine comprising a carrier and a recombinant virus as claimed in claim 3.

145

      21.   A vaccine for inducing an immunological response in a host animal inoculated with said vaccine, said vaccine comprising a carrier and a recombinant virus as claimed in claim 14.

FIG.  I

FIG.    2

3/9

VACCINIA
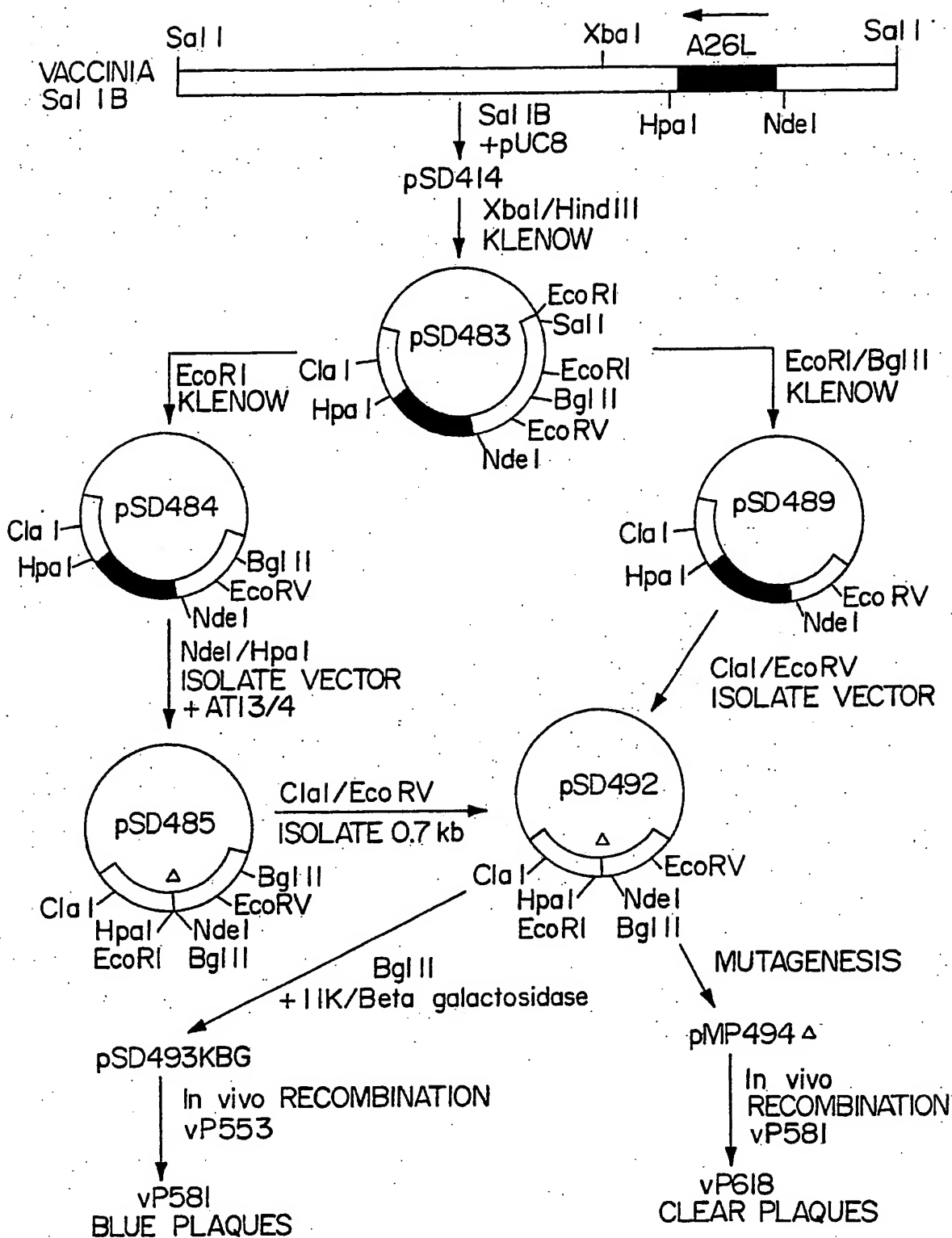Sal IB

Sal I ——— Xba I —— A26L —— Sal I
Hpa I    Nde I

Sal IB
+pUC8

pSD414

Xbal/HindIII
KLENOW

pSD483
EcoRI
Sal I
EcoRI
Bgl II
EcoRV
Cla I
Hpa I
Nde I

EcoRI
KLENOW

EcoRI/Bgl II
KLENOW

pSD484
Cla I
Hpa I
Bgl II
EcoRV
Nde I

pSD489
Cla I
Hpa I
EcoRV
Nde I

NdeI/HpaI
ISOLATE VECTOR
+ ATI 3/4

ClaI/EcoRV
ISOLATE VECTOR

pSD485
Cla I
Hpa I
EcoRI
Nde I
Bgl II
EcoRV
△

ClaI/EcoRV
ISOLATE 0.7 kb

pSD492
Cla I
Hpa I
EcoRI
Nde I
Bgl II
EcoRV
△

Bgl II
+ 11K/Beta galactosidase

MUTAGENESIS

pSD493KBG

pMP494 △

In vivo RECOMBINATION
vP553

In vivo
RECOMBINATION
vP581

vP581
BLUE PLAQUES

vP618
CLEAR PLAQUES

# FIG. 3

FIG. 4

FIG.    5

6/9



FIG.  6

FIG. 7



FIG. 8

FIG. 9



FIG. 10

FIG. 11

# INTERNATIONAL SEARCH REPORT

| International application No. |
| --- |
| PCT/US92/05107 |

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 7/00; A61K 39/12
US CL :435/235.1; 424/89

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/235.1, 236, 172.3; 424/89

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| --- | --- | --- |
| X<br>Y | Proceedings of the National Academy of Sciences USA, Volume 86, issued February 1989, D. Rodriguez et al., "Highly Attenuated Vaccinia Virus Mutants for the Generation of Safe Recombinant Viruses", pp. 1287-1291, see entire document. | 1-4,6,8,10<br>5,7,9,11-21 |
| X<br>Y | Virology, Volume 173, No.1, issued November 1989, S. Dallo et al., "Humoral Immune Response Elicited by Highly Attenuated Variants of Vaccinia Virus and by an Attenuated Recombinant Expressing HIV-1 Envelope Protein", pp. 323-328, see entire document. | 1-4,6,8,10<br>5,7,9,11-21 |
| Y | WO, A, 90/12101 (Smith) 18 October 1990, see entire document. | 1-21 |
| Y | Virology, Volume 152, issued 1986, M.E. Perkus et al., "Insertion and Deletion Mutants of Vaccinia Virus", pp. 285-297, see entire document. | 1-21 |
| Y | Virology, Volume 179, No. 1, issued November 1990, M. E. Perkus et al., "Vaccinia Virus Host Range Genes", pp. 276-286, see entire document. | 1-21 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| --- | --- | --- | --- |
| "A" | document defining the general state of the art which is not considered to be part of particular relevance | | |
| "E" | earlier document published on or after the international filing date | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | "&" | document member of the same patent family |

| Date of the actual completion of the international search | Date of mailing of the international search report |
| --- | --- |
| 09 SEPTEMBER 1992 | 1 8 SEP 1992 |

| Name and mailing address of the ISA/<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Authorized officer<br><br>D. BARND |
| --- | --- |
| Facsimile No. NOT APPLICABLE | Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Nature, Volume 317, issued 31 October 1985, R. M. L. Buller et al., "Decreased Virulence of Recombinant Vaccinia Virus Expression Vectors is Associated With a Thymidine Kinase-Negative Phenotype", pp. 813-815, see entire document. | 1-21 |
| Y | Journal of Virology, Volume 62, No. 12, issued December 1988, H. Shida et al., "Effects and Virulences of Recombinant Vaccinia Viruses Derived From Attenuated Strains That Express the Human T-Cell Leukemia Virus Type I Envelope Gene", pp. 4474-4480, see entire document. | 1-21 |
| Y | Critical Reviews in Immunology, Volume 10, No. 1, issued 1990, J. Tartaglia et al., "Poxvirus-Based Vectors as Vaccine Candidates", pp. 13-30, see entire document. | 1-21 |
| Y | WO, A, 90/10693 (Paoletti et al.) 20 September 1990, see entire document. | 1-21 |
| Y | Proceedings of the National Academy of Sciences USA, Volume 86, issued September 1989, S. Hu et al., "Protection of Macaques Against Simian AIDS by Immunization With a Recombinant Vaccinia Virus Expressing the Envelope Glycoproteins of Simian Type D Retrovirus", pp. 7213-7217, see entire document. | 12,13 |
| Y | Vaccine, Volume 6, issued December 1988, J. Taylor et al., "Fowlpox Virus as a Vector in Non-Avian Species, pp. 466-467, see entire document. | 14-19,21 |
| Y | Vaccine, Volume 6, issued December 1988, J. Taylor et al., "Recombinant Fowlpox Virus Inducing Protective Immunity in Non-Avian Species", pp. 497-503, see entire document. | 14-19,21 |
| Y | WO, A, 89/03429 (Paoletti) 20 April 1989, see entire document. | 14-19,21 |
| Y,P | Virology, Volume 188, No. 1, issued May 1992, J. Tartaglia et al., "NYVAC: A Highly Attenuated Strain of Vaccinia Virus", pp. 217-232, see entire document. | 1-21 |

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CA, MEDLINE, BIOSIS, WPI
search terms: poxvirus, vaccinia, fowlpox, canarypox, avipox, attenual?, deletion, insertion?, NYVAC, ALVAC, immunodeficiency virus, HIV, SIV, vaccine